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(54) Title: **UBIQUITIN LIGASES, AND USES RELATED THERETO**

(57) Abstract

The present invention relates to the discovery in eukaryotic cells of ubiquitin ligases. These proteins are referred to herein collectively as "pub" proteins for Protein UBiquitin ligase, and individually as h-pub1, h-pub2, h-pub3 and s-pub1 for the human pub1, pub2 and pub3 and *Schizosaccharomyces pombe* pub1 clones, respectively. *Pub1* proteins apparently play a role in the ubiquitination of the mitotic activating tyrosine phosphatase cdc25, and thus they may regulate the progression of proliferation in eukaryotic cells by activating the cyclin dependent kinase complexes. In *S. pombe*, disruption of s-pub1 elevates the level of cdc25 protein *in vivo* increasing the activity of the tyrosine kinases, wee1 and mik1, required to arrest the cell-cycle. Loss of wee1 function in an *S. pombe* cell carrying a disruption in the s-pub1 gene results in a lethal premature entry into mitosis; such lethal phenotype can be rescued by the loss of cdc25 function. A ubiquitin thioester adduct of s-pub1 can be isolated from *S. pombe* and disruption of s-pub1 dramatically reduces ubiquitination of cdc25.

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Ubiquitin Ligases, and Uses Related Thereto**Background of the Invention****(a) Cell-cycle regulatory proteins**

5 Cell-cycle events are thought to be regulated by a series of interdependent biochemical steps. In eukaryotic cells mitosis does not normally take place until the G1, S and G2 phases of the cell-cycle are completed. In all eukaryotic cells examined to date, the cell cycle appears to be regulated by the sequential activation of a series of the CDK's or Cyclin Dependent Kinases (reviewed in Morgan, (1995) *Nature* 374:131-134; King et al.,
10 (1994) *Cell* 79:563-571; Norbury and Nurse, (1992) *Annu. Rev. Biochem.* 61:441-470). Yeast cells contain a single CDK known as cdc2 in *S. pombe* (Beach et al., (1982) *Nature* 300:706-709; Booher and Beach, (1986) *Gene* 31:129-134; Hindley and Phear, (1984) *Gene* 21:129-134; Nurse and Bissett, (1981) *Nature* 292:558-560; Simanis and Nurse, (1986) *Cell* 45:261-268; and for review see Forsburg and Nurse, (1991b) *Annu. Rev. Cell Biol.* 7:227-
15 256) and cdc28 in *S. cerevisiae*. The similarities between the progression of proliferation in mammalian cells and yeast have suggested similar roles for cdc protein kinases across species. In support of this hypothesis, a human cdc2 gene has been found to be able to substitute for the activity of an *S. pombe* cdc2 gene in both its G1/S and G2/M roles (Lee et al., (1987) *Nature* 327:31). Likewise, the fact that the cdc2 homolog of *S. cerevisiae* (cdc28)
20 can be replaced by the human cdc2 also emphasizes the extent to which the basic cell-cycle machinery has been conserved in evolution.

The activation of cdc2 kinase activity occurs during the M phase and is controlled at multiple levels involving, among other events, the association with various cyclin subunits and the phosphorylation on threonine 167 by cdc2 activating kinase (CAK) (Booher and Beach, (1987) *EMBO J.* 6:3441-3447; Booher et al., (1989) *Cell* 58:485-497; Bueno et al.,
25 (1991) *Cell* 66:149-159; Bueno and Russell, (1993) *Mol. Cell Biol.* 13:2286-2297; Connolly and Beach, (1994) *Mol. Cell Biol.* 14:768-776; Fesquet et al., (1993) *EMBO J.* 12:3111-3121; Forsburg and Nurse, (1991a) *Nature* 351:245-247; Gould et al., (1991) *EMBO J.* 3297-3309; Hagan et al., (1988) *J. Cell Sci.* 91:587-595; Solomon et al., (1992) *Mol. Biol. Cell* 3:13-27; Solomon et al., (1993) *EMBO J.* 12:3133-3142). Another well-characterized mechanism of regulating the activity of cdc2 involves its inhibition by phosphorylation of a tyrosine and threonine residues (Tyr-15 and Thr-14) within its ATP binding site (Gould and Nurse, (1989) *supra*). The inhibitory phosphorylation of cdc2 is mediated at least in part by the wee1 and mik1 tyrosine kinases (Russel et al., (1987) *Cell* 49:559-567; Lundgren et al.,
30 (1991) *Cell* 64:1111-1122; Featherstone et al., (1991) *Nature* 349:808-811; and Parker et al., (1992) *PNAS* 89:2917-2921). These kinases act as mitotic inhibitors, over-expression of them causes cells to arrest in the G2 phase of the cell-cycle. By contrast, loss of function of wee1 causes a modest advancement of mitosis, whereas loss of both wee1 and mik1
35

function causes grossly premature mitosis, uncoupled from all checkpoints that normally restrain cell division (Lundgren et al., (1991) *Cell* 64:1111-1122).

As the cell is about to reach the end of G2, dephosphorylation of the cdc2-inactivating Thr-14 and Tyr-15 residues occurs leading to activation of the cdc2 complex as a kinase. With the exception of budding yeast and the early embryonic cell divisions of some organisms, the dephosphorylation of tyrosine 15 is a key regulatory step of cdc2 activation (Morla et al., (1989) *Cell* 58:193-203; Heald et al., (1993) *Cell* 74:463-474; and for reviews see King et al., (1994) *Cell* 79:563-571; and Morgan (1995) *Nature* 374:131-134). A stimulatory phosphatase, known as cdc25, is responsible for Tyr-15 and Thr-14 dephosphorylation and serves as a rate-limiting mitotic activator. (Dunphy et al., (1991) *Cell* 67:189-196; Lee et al., (1992) *Mol Biol Cell* 3:73-84; Millar et al., (1991) *EMBO J* 10:4301-4309; and Russell et al., (1986) *Cell* 45:145-153). Cdc25 has been shown to be required for entry into mitosis in a number of different organisms (King et al., 1994). Evidence indicates that both the cdc25 phosphatase and the cdc2-specific tyrosine kinases are detectably active during interphase, suggesting that there is an ongoing competition between these two activities prior to mitosis (Kumagai et al., (1992) *Cell* 70:139-151; Smythe et al., (1992) *Cell* 68:787-797; and Solomon et al., (1990) *Cell* 63:1013-1024. This situation implies that the initial decision to enter mitosis involves a modulation of the equilibrium of the phosphorylation state of cdc2 which is likely controlled by variation of the rate of tyrosine dephosphorylation of cdc2 and/or a decrease in the rate of its tyrosine phosphorylation.

In *S. pombe*, the level of cdc25 oscillates in a cell cycle dependent fashion (Ducommum et al., (1990) *Biochem. Biophys. Res. Comm.* 167:301-309; Moreno et al., (1990) *Nature* 344:549-552). Cdc25 accumulates through the cell cycle until mitosis when its level rapidly decreases. The pattern of cdc25 accumulation during the cell cycle is reminiscent of mitotic cyclins which are degraded by the ubiquitin system (Glotzer et al., (1991) *Nature* 349:132-138; Seufert et al., (1995) *Nature* 373:78-81).

(b) Ubiquitination pathways

The ubiquitin-mediated proteolysis system is the major pathway for the selective, controlled degradation of intracellular proteins in eukaryotic cells. Ubiquitin modification of a variety of protein targets within the cell appears to be important in a number of basic cellular functions such as regulation of gene expression, regulation of the cell-cycle, modification of cell surface receptors, biogenesis of ribosomes, and DNA repair. One major function of the ubiquitin-mediated system is to control the half-lives of cellular proteins. The half-life of different proteins can range from a few minutes to several days, and can vary considerably depending on the cell-type, nutritional and environmental conditions, as well as the stage of the cell-cycle.

Targeted proteins undergoing selective degradation, presumably through the actions of a ubiquitin-dependent proteosome, are covalently tagged with ubiquitin through the

formation of an isopeptide bond between the C-terminal glycyl residue of ubiquitin and a specific lysyl residue in the substrate protein. This process is catalyzed by a ubiquitin-activating enzyme (E1) and a ubiquitin-conjugating enzyme (E2), and in some instances may also require auxiliary substrate recognition proteins (E3s). Following the linkage of the first 5 ubiquitin chain, additional molecules of ubiquitin may be attached to lysine side chains of the previously conjugated moiety to form branched multi-ubiquitin chains.

The conjugation of ubiquitin to protein substrates is a multi-step process. In an initial ATP requiring step, a thioester is formed between the C-terminus of ubiquitin and an internal cysteine residue of an E1 enzyme. Activated ubiquitin is then transferred to a specific 10 cysteine on one of several E2 enzymes. Finally, these E2 enzymes donate ubiquitin to protein substrates. Substrates are recognized either directly by ubiquitin-conjugated enzymes or by associated substrate recognition proteins, the E3 proteins, also known as ubiquitin ligases.

Many proteins that control cell-cycle progression are short-lived. For example, 15 regulation of oncoproteins and anti-oncoproteins clearly plays an important role in determining steady-state levels of protein expression, and alterations in protein degradation are as likely as changes in transcription and/or translation to cause either the proliferative arrest of cells, or alternatively, the transformation of cells.

20 *Summary of the Invention*

The present invention relates to the discovery in eukaryotic cells of novel family of proteins whose apparent function includes a ubiquitin ligase activity. In particular, one feature of members of this family of proteins includes a catalytic domain containing a region homologous to the putative catalytic domain of the human protein ubiquitin ligase E6-AP. 25 The subject proteins are referred to herein collectively as "pub proteins" or "pub ligases" for Protein UBiquitin ligase. As described herein, this family of proteins include at least two paralogous classes of mammalian homologs, "pub1" and "pub2". We have cloned at least one human pub1 gene (h-pub1), e.g., a human *pub1* protein having an apparent molecular weight of about 84.5 kDa, as well as a *Schizosaccharomyces pombe* pub1 gene (s-pub1) 30 having an apparent molecular weight of about 85 kDa. Additionally, we have cloned a human pub2 gene (h-pub2) characterized by an apparent molecular weight of about 96.2kd, and a human pub3 gene (h-pub3) characterized by an apparent molecular weight of about 91.7kd. The *pub* proteins have an apparent function in the ubiquitination of, among other cellular proteins, the mitotic activating tyrosine phosphatase cdc25 and the tumor suppressor 35 protein p53. Accordingly, the subject proteins may be involved in regulating the progression of proliferation in eukaryotic cells by effectively controlling the activity of the cdk complexes by modulating the availability of cdc25 and/or p53.

Moreover, as described in further detail below, the subject *pub1* proteins contain a sequence motif (CaLB) which is highly homologous to a consensus sequence which has been

implicated in Ca^{+2} -dependent binding to phospholipid membranes in several proteins such as phospholipase A2, PKC and rasGAP.

5 In *S. pombe*, disruption of s-pub1 elevates the level of cdc25 protein *in vivo* increasing the activity of the tyrosine kinases, wee1 and mik1, required to arrest the cell cycle. Loss of wee1 function in an *S. pombe* cell carrying a disruption in the s-pub1 gene results in a lethal premature entry into mitosis; such lethal phenotype can be rescued by the loss of cdc25 function. An ubiquitin thioester adduct of s-pub1 can be isolated from *S. pombe* and disruption of s-pub1 dramatically reduces ubiquitination of cdc25. These results indicate that s-pub1 may directly ubiquitinate cdc25 *in vivo*.

10 One aspect of the invention features a substantially pure preparation of an h-pub1 polypeptide, e.g., full length or fragments thereof, the full-length form of the h-pub1 protein having an approximate molecular weight in the range of 75-95 kD, preferably about 80-90 kD. In a preferred embodiment: the polypeptide has an amino acid sequence at least 70% homologous to an amino acid sequence represented in SEQ ID No. 2; the polypeptide has an 15 amino acid sequence at least 80% homologous to an amino acid sequence represented in SEQ ID No. 2; the polypeptide has an amino acid sequence at least 90% homologous to an amino acid sequence represented in SEQ ID No. 2; the polypeptide has an amino acid sequence identical to an amino acid sequence represented in SEQ ID No. 2. In preferred embodiments the fragment comprises at least, for example, 25, 50 or 75 contiguous amino 20 acid residues of SEQ ID No. 2. For instance, certain embodiments of the subject h-pub1 protein will include a catalytic domain having a ubiquitin ligase activity, and (optionally) all or only a portion of other sequences of the full-length h-pub1, e.g. a calcium-binding domain (CalB motif) and/or an ATP-binding site.

25 Another aspect of the invention features a substantially pure preparation of an h-pub2 polypeptide, e.g., full length or fragments thereof, the full-length form of the h-pub2 protein having an approximate molecular weight in the range of 85-105 kD, preferably about 90-100 kD. In a preferred embodiment: the polypeptide has an amino acid sequence at least 70% homologous to an amino acid sequence represented in SEQ ID No. 6; the polypeptide has an 30 amino acid sequence at least 80% homologous to an amino acid sequence represented in SEQ ID No. 6; the polypeptide has an amino acid sequence at least 90% homologous to an amino acid sequence represented in SEQ ID No. 6; the polypeptide has an amino acid sequence identical to an amino acid sequence represented in SEQ ID No. 6. In preferred embodiments the fragment comprises at least, for example, 25, 50 or 75 contiguous amino acid residues of SEQ ID No. 6. For instance, certain embodiments of the subject h-pub2 35 protein will include a catalytic domain having a ubiquitin ligase activity.

Yet another aspect of the invention features a substantially pure preparation of an h-pub3 polypeptide, e.g., full length or fragments thereof, the full-length form of the h-pub3 protein having an approximate molecular weight in the range of 80-100 kD, preferably about 85-95 kD. In a preferred embodiment: the polypeptide has an amino acid sequence at least

70% homologous to an amino acid sequence represented in SEQ ID No. 8; the polypeptide has an amino acid sequence at least 80% homologous to an amino acid sequence represented in SEQ ID No. 8; the polypeptide has an amino acid sequence at least 90% homologous to an amino acid sequence represented in SEQ ID No. 8; the polypeptide has an amino acid sequence identical to an amino acid sequence represented in SEQ ID No. 8. In preferred embodiments the fragment comprises at least, for example, 25, 50 or 75 contiguous amino acid residues of SEQ ID No. 8. For instance, certain embodiments of the subject h-pub3 protein will include a catalytic domain having a ubiquitin ligase activity.

Still another aspect of the invention features a substantially pure preparation of an s-pub1 polypeptide, including fragments of the full-length portion, the full-length form of the p85 protein having an approximate molecular weight in the range of 80-90 kD, preferably about 85 kD. In a preferred embodiment: the polypeptide has an amino acid sequence at least 70% homologous to an amino acid sequence represented in SEQ ID No. 4; the polypeptide has an amino acid sequence at least 80% homologous to an amino acid sequence represented in SEQ ID No. 4; the polypeptide has an amino acid sequence at least 90% homologous to an amino acid sequence represented in SEQ ID No. 4; the polypeptide has an amino acid sequence identical to an amino acid sequence represented in SEQ ID No. 4. In preferred embodiments: the fragment comprises at least 25, 50 or 75 contiguous amino acid residues of SEQ ID No. 4. As above, preferred embodiments of the subject s-pub1 protein include a catalytic domain and (optionally) a Calb motif and/or ATP-binding site. However, it will be understood that, for certain uses, only the non-catalytic domains/motifs may be desired.

Polypeptides referred to herein as *pub* polypeptides, in addition to h-pub1, h-pub2, h-pub3 or s-pub1 further refers to other mammalian paralogs, or other mammalian orthologs.

In general, the biological activity of a *pub* polypeptide can be characterized as including the ability to transfer an ubiquitin molecule from the relevant ubiquitin conjugating enzyme (UBC) to a residue of a target through a *pub* ubiquitin thioester intermediate. Moreover, a "pub biological activity" also refers to an ability to specifically antagonize the biochemical action of a wild-type *pub* protein, e.g., a *pub* protein represented by SEQ ID Nos. 2, 4, 6 and/or 8. In other words, dominant negative mutants of *pub* are included within the scope of *pub* biological activity. Such mutants are exemplified by mutation of the active site cysteine to an alanine or other catalytically inactivating mutant. The biological activity of the *pub1* proteins may also include the ability to translocate to specific phospholipid membranes in the presence of calcium and/or to bind a nucleotidyl phosphate such as ATP.

The above notwithstanding, the biological activity of a *pub* polypeptide may be characterized by one or more of the following attributes: an ability to regulate the cell-cycle of an eukaryotic cell; an ability to modulate proliferation/cell growth of an eukaryotic cell; an ability to modulate entry of a mammalian or yeast cell into M phase; an ability to ubiquitinate a cell-cycle regulator, such as, e.g. a tyrosine phosphatase involved in cell-cycle

progression, e.g. a cdc25 phosphatase, or a tumor suppressor protein, e.g., p53. Such activities may be manifested by the ability to control the steady state level of cdc25 phosphatase, and thus to control the degree of dephosphorylation of a CDK kinase, e.g. cdc2 or the like. Such activities may also be manifested by the ability to control the steady state 5 level of p53, and thus to control the degree of cell-cycle regulation by that protein. The *pub* polypeptides of the present invention may also function to modulate differentiation of cells/tissue. The subject polypeptides of this invention may also be capable of modulating cell growth or proliferation by influencing the action of other cellular proteins. A *pub* polypeptide can be a specific agonist of the function of the wild-type form of the protein, or 10 can be a specific antagonist.

Yet another aspect of the present invention concerns an immunogen comprising a *pub* polypeptide of the present invention, or a fragment thereof, in an immunogenic preparation, the immunogen being capable of eliciting an immune response specific for the *pub* polypeptide; e.g. a humoral response, e.g. an antibody response; e.g. a cellular response.

15 Another aspect of the present invention features recombinant h-pub1, h-pub2, h-pub3, h-pub4 or s-pub1 polypeptides, or fragments thereof, having amino acid sequences preferably identical or homologous to the amino acid sequence designated by SEQ ID No. 2, SEQ ID No. 4, SEQ ID No. 6 or SEQ ID No. 8, respectively.

Another aspect of the present invention provides a substantially pure nucleic acid 20 having a nucleotide sequence which encodes an h-pub1 polypeptide, or a fragment thereof, having an amino acid sequence at least 70% homologous to SEQ ID No. 2. In a more preferred embodiment: the nucleic acid encodes a protein having an amino acid sequence at least 80% homologous to SEQ ID No. 2, more preferably at least 90% homologous to SEQ ID No. 2, and most preferably at least 95% homologous to SEQ ID No. 2. The nucleic 25 preferably encodes an h-pub1 protein which specifically transfers an ubiquitin molecule from the relevant UBC to a substrate protein, e.g., cdc25, or specifically antagonizes such ubiquitination.

Another aspect of the present invention provides a substantially pure nucleic acid having a nucleotide sequence which encodes an h-pub2 polypeptide, or a fragment thereof, 30 having an amino acid sequence at least 70% homologous to SEQ ID No. 6. In a more preferred embodiment: the nucleic acid encodes a protein having an amino acid sequence at least 80% homologous to SEQ ID No. 6, more preferably at least 90% homologous to SEQ ID No. 6, and most preferably at least 95% homologous to SEQ ID No. 6. The nucleic preferably encodes an h-pub2 protein which specifically transfers an ubiquitin molecule 35 from the relevant UBC to a substrate protein, e.g., cdc25, or specifically antagonizes such ubiquitination.

Still another aspect of the present invention provides a substantially pure nucleic acid having a nucleotide sequence which encodes an h-pub3 polypeptide, or a fragment thereof, having an amino acid sequence at least 70% homologous to SEQ ID No. 8. In a more

preferred embodiment: the nucleic acid encodes a protein having an amino acid sequence at least 80% homologous to SEQ ID No. 8, more preferably at least 90% homologous to SEQ ID No. 8, and most preferably at least 95% homologous to SEQ ID No. 8. The nucleic acid preferably encodes an h-pub3 protein which specifically transfers an ubiquitin molecule from the relevant UBC to a substrate protein, e.g., p53 and/or cdc25.

Yet another aspect of the present invention provides a substantially pure nucleic acid having a nucleotide sequence which encodes an s-pub1 polypeptide, or a fragment thereof, having an amino acid sequence at least 70% homologous to SEQ ID No. 4. In a more preferred embodiment: the nucleic acid encodes a protein having an amino acid sequence at least 80% homologous to SEQ ID No. 4, more preferably at least 90% homologous to SEQ ID No. 4, and most preferably at least 95% homologous to SEQ ID No. 4. The nucleic acid preferably encodes an s-pub1 protein which specifically transfers an ubiquitin molecule from the relevant UBC to a cell cycle regulator, e.g., mitotic activating tyrosine phosphatase, e.g., cdc25.

In another embodiment, the nucleic acid hybridizes under stringent conditions to a nucleic acid probe corresponding to at least 25 consecutive nucleotides of SEQ ID Nos. 1, 3, 5 or 7; more preferably to at least 50 consecutive nucleotides of one or both of SEQ ID Nos. 1, 3, 5 or 7; more preferably to at least 75 consecutive nucleotides of SEQ ID No. 1, 3, 5 or 7.

Furthermore, in certain embodiments, the *pub* nucleic acid will comprise a transcriptional regulatory sequence, e.g. at least one of a transcriptional promoter or transcriptional enhancer sequence, operably linked to the *pub* gene sequence so as to render the recombinant *pub* gene sequence suitable for use as an expression vector.

The present invention also features transgenic non-human animals, e.g. mice, which either express a heterologous *pub* gene, e.g. derived from humans, or which mis-express their own *pub* gene, e.g. expression is disrupted. Such a transgenic animal can serve as an animal model for studying cellular disorders comprising mutated or mis-expressed *pub* alleles.

The present invention also provides a probe/primer comprising a substantially purified oligonucleotide, wherein the oligonucleotide comprises a region of nucleotide sequence which hybridizes under stringent conditions to at least 10 consecutive nucleotides of sense or antisense sequence of SEQ ID Nos. 1, 3, 5 or 7, or naturally occurring mutants thereof. In preferred embodiments, the probe/primer further comprises a label group attached thereto and able to be detected, e.g. the label group is selected from a group consisting of radioisotopes, fluorescent compounds, enzymes, and enzyme co-factors. Such probes can be used as a part of a diagnostic test kit for identifying transformed cells, such as for measuring a level of a nucleic acid encoding a *pub* polypeptide in a sample of cells isolated from a patient; e.g. for measuring the mRNA level in a cell or determining whether the genomic *pub* gene has been mutated or deleted.

Another aspect of the present invention provides a method of determining if a subject, e.g. a human patient, is at risk for a disorder characterized by unwanted cell proliferation, comprising detecting, in a tissue of the subject, the presence or absence of a genetic lesion characterized by at least one of (i) a mutation of a *pub* gene, e.g., encoding a 5 *pub1* protein represented by SEQ ID No. 2, a *pub2* protein represented by SEQ ID No. 6, a *pub3* protein represented by SEQ ID No. 8, or a homolog thereof; (ii) the mis-expression of the *pub* gene. In preferred embodiments: detecting the genetic lesion comprises ascertaining the existence of at least one of a deletion of one or more nucleotides from said gene, an 10 addition of one or more nucleotides to said gene, an substitution of one or more nucleotides of said gene, a gross chromosomal rearrangement of said gene, a gross alteration in the level of a messenger RNA transcript of said gene, the presence of a non-wild type splicing pattern of a messenger RNA transcript of said gene, or a non-wild type level of said protein. For 15 example, detecting the genetic lesion can comprise (i) providing a probe/primer comprising an oligonucleotide containing a region of nucleotide sequence which hybridizes to a sense or antisense sequence of SEQ ID No. 1, 5 or 7, or naturally occurring mutants thereof, or 5' or 3' flanking sequences naturally associated with the *pub* gene; (ii) exposing the probe/primer to nucleic acid of the tissue; and (iii) detecting, by hybridization of the probe/primer to the nucleic acid, the presence or absence of the genetic lesion; e.g. wherein detecting the lesion comprises utilizing the probe/primer to determine the nucleotide sequence of the *pub* gene 20 and, optionally, of the flanking nucleic acid sequences; e.g. wherein detecting the lesion comprises utilizing the probe/primer in a polymerase chain reaction (PCR); e.g. wherein detecting the lesion comprises utilizing the probe/primer in a ligation chain reaction (LCR). In alternate embodiments, the level of said protein is detected in an immunoassay.

Moreover, the present invention provides a practical approach for the identification 25 of candidate agents able to modulate, e.g., activate or inhibit, ubiquitin-mediated degradation of a cell-cycle regulatory protein in eukaryotic cells, especially yeast and mammalian cells. For instance, the assays permit identification of agents which modulate the ubiquitination of a cell cycle regulatory protein, e.g., a mitotic activating tyrosine phosphatase such as a *cdc25* phosphatase, or e.g., a tumor suppressor such as *p53*.

One aspect of the present invention relates to a method for identifying an activator or 30 an inhibitor of ubiquitin-mediated proteolysis of a cell-cycle regulatory protein by (i) providing a ubiquitin-conjugating system that includes the substrate protein, an E3-like complex (e.g., comprising a *pub* protein a ligase activity thereof), and ubiquitin under conditions which promote the ubiquitination of the target protein, and (ii) measuring the 35 level of ubiquitination of the subject protein brought about by the system in the presence and absence of a candidate agent. For example, a decrease in the level of ubiquitin conjugation is indicative of an inhibitory activity for the candidate agent. The level of ubiquitination of the regulatory protein can be measured by determining the actual concentration of protein:ubiquitin conjugates formed; or inferred by detecting some other quality of the

subject protein affected by ubiquitination, including the proteolytic degradation of the protein. In certain embodiments, the present assay comprises an *in vivo* ubiquitin-conjugating system, such as a cell able to conduct the regulatory protein through at least a portion of a ubiquitin-mediated proteolytic pathway. In other embodiments, the present 5 assay comprises an *in vitro* ubiquitin-conjugating system comprising a reconstituted protein mixture in which at least the ability to transfer ubiquitin to the regulatory protein is constituted.

Still another approach relies on a competitive binding assay to detect potential 10 modulatory agents. For example, the ability of all or a portion of the *pub* protein to bind to cdc25, p53 or another cellular substrate protein, or other components of the ubiquitin pathway (e.g. E2's) can be assessed in the presence and absence of a test agent. In similar 15 fashion, the ability of a test agent to modulate the function of the CaLB motif of a *pub1* protein can be assessed.

The present invention also provides a method for producing a hyper- or a hypo- 20 proliferative cell, e.g., a cell which has an impaired cell-cycle checkpoint such as the premature progression of the cell through at least a portion of a cell-cycle. As an example, a hyper-proliferative cell, e.g., a transformed mammalian cell, can be produced by disrupting a *pub* gene or gene product. Such cells are useful for identifying agents that modulate 25 proliferation such as mitotic inhibitors, e.g., agents which may inhibit at least one regulatory protein of the cell cycle in a manner which counter-balances the effect of the impairment.

The impaired checkpoint can be generated, for example, by molecular biological, 30 genetic, and/or biochemical means. The checkpoint to be impaired can comprise a regulatory protein or proteins which control progression through the cell-cycle, such as those which control the G2/M transition. By way of example, the impaired checkpoint can 35 comprise a *pub* protein which controls the ubiquitination of a cdc25 phosphatase, and thus the degree of dephosphorylation of a CDK protein kinase, such as cdc2. In another example, the impaired checkpoint can comprise a *pub* protein which controls the ubiquitination of p53, and thus the progression of the cell through certain cell-cycle checkpoints, such as responsiveness to DNA damage.

In another embodiment, cells impaired in a mitotic checkpoint can also be created by 30 using agents which disrupt the binding of a *pub* protein to at least one of its targets, e.g., a cdc25 phosphatase. Such a system can be used to modulate cell proliferation and/or growth. In one embodiment, the method comprises administering a *pub* mimetic, e.g. a peptidomimetic, which binds to a cdc25 phosphatase or p53, and inhibits the interaction 35 between that protein and a *pub* ligase.

Furthermore, humanized yeast cells can be generated so as to comprise heterologous 30 cell-cycle proteins (i.e. cross-species expression). For example, an exogenous *pub* can be expressed in a *Schizosaccharomyces* cell, such as *Schizosaccharomyces pombe* carrying a null mutation of the *pub* gene. The exogenous *pub* can be, for example, a human *pub*

homolog described herein. Humanized yeast cells can provide useful assays for screening modulators, e.g., activators or inhibitors, of proliferation *in vivo*.

Other features and advantages of the invention will be apparent from the following detailed description, and from the claims. The practice of the present invention will employ, unless otherwise indicated, conventional techniques of cell biology, cell culture, molecular biology, transgenic biology, microbiology, recombinant DNA, and immunology, which are within the skill of the art. Such techniques are explained fully in the literature. See, for example, *Molecular Cloning A Laboratory Manual*, 2nd Ed., ed. by Sambrook, Fritsch and Maniatis (Cold Spring Harbor Laboratory Press, 1989); *DNA Cloning*, Volumes 5 I and II (D. N. Glover ed., 1985); *Oligonucleotide Synthesis* (M. J. Gait ed., 1984); Mullis et al., U.S. Patent No. 4,683,195; *Nucleic Acid Hybridization* (B. D. Hames & S. J. Higgins eds. 1984); *Transcription And Translation* (B. D. Hames & S. J. Higgins eds. 1984); *Culture Of Animal Cells* (R. I. Freshney, Alan R. Liss, Inc., 1987); *Immobilized Cells And Enzymes* (IRL Press, 1986); B. Perbal, *A Practical Guide To Molecular Cloning* (1984); the treatise, 10 *Methods In Enzymology* (Academic Press, Inc., N.Y.); *Gene Transfer Vectors For Mammalian Cells* (J. H. Miller and M. P. Calos eds., 1987, Cold Spring Harbor Laboratory); *Methods In Enzymology*, Vols. 154 and 155 (Wu et al., eds.), *Immunochemical Methods In Cell And Molecular Biology* (Mayer and Walker, eds., Academic Press, London, 1987); *Handbook Of Experimental Immunology*, Volumes I-IV (D. M. Weir and C. C. Blackwell, 15 eds., 1986); *Manipulating the Mouse Embryo*, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1986).

Detailed Description of the Invention

The cyclin dependent kinases are subject to multiple levels of control. One well-characterized mechanism regulating the activity of cdks involves the phosphorylation of tyrosine, threonine, and serine residues; the phosphorylation level of which varies during the cell-cycle (Draetta et al. (1988) *Nature* 336:738-744; Dunphy et al. (1989) *Cell* 58:181-191; Morla et al. (1989) *Cell* 58:193-203; Gould et al. (1989) *Nature* 342:39-45; and Solomon et al. (1990) *Cell* 63:1013-1024). The phosphorylation of cdc2, for example, on Tyr-15 and 20 Thr-14, two residues located in the putative ATP binding site of the kinase, negatively regulates kinase activity. This inhibitory phosphorylation of cdc2 is mediated at least in part by the wee1 and mik1 tyrosine kinases (Russel et al. (1987) *Cell* 49:559-567; Lundgren et al. (1991) *Cell* 64:1111-1122; Featherstone et al. (1991) *Nature* 349:808-811; and Parker et al. (1992) *PNAS* 89:2917-2921). These kinases act as mitotic inhibitors, over-expression of 25 which causes cells to arrest in the G2 phase of the cell-cycle. By contrast, loss of function of wee1 causes a modest advancement of mitosis, whereas loss of both wee1 and mik1 function causes grossly premature mitosis, uncoupled from all checkpoints that normally restrain cell division (Lundgren et al. (1991) *Cell* 64:1111-1122).

Dephosphorylation of the cdk-inactivating Thr-14 and Tyr-15 residues occurs leading to activation of the cdk/cyclin complex as a kinase. A stimulatory phosphatase, known as cdc25, is responsible for Tyr-15 and Thr-14 dephosphorylation and serves as a rate-limiting mitotic activator. (Dunphy et al. (1991) *Cell* 67:189-196; Lee et al. (1992) *Mol Biol Cell* 3:73-84; Millar et al. (1991) *EMBO J* 10:4301-4309; and Russell et al. (1986) *Cell* 45:145-153). Recent evidence indicates that both the cdc25 phosphatase and the cdk-specific tyrosine kinases (wee1/mik1) are detectably active during the cell-cycle, suggesting that there is an ongoing competition between these two activities to fine tune cell-cycle progression (Kumagai et al. (1992) *Cell* 70:139-151; Smythe et al. (1992) *Cell* 68:787-797; 10 and Solomon et al. (1990) *Cell* 63:1013-1024.

The role of the ubiquitin dependent proteolytic pathway in the regulation of cdc25 has been examined by us both *in vivo* and *in vitro*. We have observed that cdc25A can be ubiquitinated *in vitro*, which ubiquitination requires an active E1 enzyme. Furthermore, we have found that the level of cdc25 protein increases upon inactivation of a temperature 15 sensitive E1 gene. In addition, poly-ubiquitinated cdc25 can be detected in cells overexpressing a histidine-tagged ubiquitin gene. Finally, inhibition of the 26S proteosome with the peptide aldehyde N-acetyl-Leu-Leu-norleucinal (LLnL) leads to the accumulation of the phosphorylated form of cdc25. Moreover, results from *in vitro* ubiquitination reactions support the notion that phosphorylation of cdc25 may be a necessary prerequisite for 20 ubiquitination. This finding is likely to be physiologically relevant to the regulated degradation of cdc25, because it is the phosphorylated form of cdc25 which is active as a protein phosphatase.

The specificity of the ubiquitination reaction is thought to be conferred at least in part by the E3 protein. We therefore sought to clone the E3 ligase(s) which specifically target 25 cdc25 for ubiquitin-dependent degradation. The present invention makes available nucleic acids encoding gene products which play a role in the ubiquitinylation of cdc25, and perhaps other regulatory proteins. Accordingly, the subject gene products may effect growth of eukaryotic cells by functioning as a tumor suppressor which down regulates mitotic 30 activation by cdc25. Given the prominence of the cdc25 regulatory pathways in various aspects of cell growth, and probably differentiation, a salient feature for each of the subject nucleic acids, polypeptides, antibodies, and derivatives thereof, includes both therapeutic and diagnostic uses. Moreover, drug screening assays are described herein which provide a systematic and practical approach for identifying candidate agents able to modulate, e.g., 35 activate or inhibit, ubiquitin-mediated degradation of a cell-cycle regulatory protein, such as the mitotic activating tyrosine phosphatase cdc25, in the eukaryotic cells, e.g. mammalian, e.g., human cells.

In particular, as described in the appended examples, the present invention describes the cloning of novel proteins containing a region homologous to the putative catalytic domain of the human protein ubiquitin ligase E6-AP and other ubiquitin ligases. The

proteins which are the subject of the present invention are referred to herein collectively as "pub" proteins for protein ubiquitin ligases. As described herein, these proteins include a yeast *pub* gene product and several human homologs. For example, we have cloned the genes for a human *pub* protein, referred to herein as "h-pub1", having an apparent molecular weight of 84.5 kDa (h-pub1), as well as a *Schizosaccharomyces pombe* homolog, "s-pub1", having an apparent molecular weight of 85 kDa. In addition, we have cloned other *pub* paralogs from human cDNA libraries, such as the 96.2 kd "h-pub2" polypeptide and the 92 kd "h-pub3" polypeptides described below. The nucleic acid and amino acid sequences, respectively, for each of the exemplary *pub* proteins are provided in the appended sequence listing as follows: SEQ ID No. 1 and 2 for h-pub1, SEQ ID No. 3 and 4 for s-pub1, SEQ ID No. 5 and 6 for h-pub2, and SEQ ID No. 7 and 8 for h-pub3.

15 *Table 1*
Guide to *Pub* sequences in Sequence Listing

	Nucleotide	Amino Acid
h-pub1	SEQ ID No. 1	SEQ ID No. 2
s-pub1	SEQ ID No. 3	SEQ ID No. 4
h-pub2	SEQ ID No. 5	SEQ ID No. 6
h-pub3	SEQ ID No. 7	SEQ ID No. 8

The overall sequence homology between the *pub* proteins is shown in Table 2.

20 *Table 2*
Amino acid sequence identity between *pub* proteins.

	h-pub1	
h-pub2	35%	h-pub2
h-pub3	28%	26%
s-pub1	38%	36%
		27%

25 It is contemplated by the present invention that the cloned *h-pub* genes set out in the appended sequence listing, in addition to representing an inter-species family of related genes, are also each part of an intra-species family. That is, it is anticipated that other paralogs of the human *pub* proteins exist, as well as orthologs of each human *pub* gene are conserved amongst other animals.

The *pub* proteins apparently play a role in the ubiquitination of regulatory proteins, such as the mitotic activating tyrosine phosphatase *cdc25* and the tumor suppressor gene *p53*, and thus they may regulate the progression of proliferation in eukaryotic cells by 30 regulating the activity of *cdk* complexes. All known protein ubiquitin ligases (E3s) contain a carboxyl terminal "hect" domain (for homologous to E6-AP carboxyl terminus). See *Huibregtse et al. (1995) PNAS 92:2563-2567*. The *hect* domain for *s-pub1* corresponds to

Tyr662-Glu766 of SEQ ID No. 4, while the hect domain of h-pub1 is provided by Ile652-Glu748 of SEQ ID No. 2, the hect domain of h-pub2 is represented in Ile739-Asp834 of SEQ ID No. 6, and the hect domain of h-pub3 is represented in Ile708-Ala804 of SEQ ID No. 8. The active site cysteine resides in the hect domain (Cys734 for s-pub1, Cys716 for h-pub1, 5 Cys801 for h-pub2, and Cys771 for h-pub3).

Both h-pub1 and h-pub2 share about 50 percent homology with the hect domain of s-pub1. The fission yeast pub1 protein apparently has two additional motifs, an ATP binding motif (Gly84-Gly89) and a calcium lipid binding domain (Leu20-Asn67; termed here a "CaLB" motif) which is highly homologous to a consensus sequence implicated in Ca^{+2} dependent binding to phospholipid membranes in several proteins such as phospholipase A2, PKC and rasGAP. Both the CaLB and ATP binding domains of s-pub1 are conserved in h-pub1 (see SEQ ID No. 2, Leu32-Ser72 for CaLB motif, and Gly90-Gly95 for ATP binding motif), but not apparently in h-pub2.

In *S. pombe*, disruption of s-pub1 elevates the level of cdc25 protein *in vivo*. Loss of 15 weel function in an *S. pombe* cell carrying a disruption in the s-pub1 gene results in a lethal premature entry into mitosis; such lethal phenotype can be rescued by the loss of cdc25 function. An ubiquitin thioester adduct of s-pub1 can be isolated from *S. pombe* and disruption of s-pub1 dramatically reduces ubiquitination of cdc25. These results suggest that 20 s-pub1 may directly ubiquitinate cdc25 *in vivo*. Human pub1 was found to complement the loss of the fission yeast gene and restore the cell size at mitosis to wild-type. This indicates that h-pub1 is a biologically active, functional homolog of yeast pub1.

For convenience, certain terms employed in the specification, examples, and appended claims are collected here.

The terms peptides, proteins and polypeptides are used interchangeably herein.

As used herein, the term "nucleic acid" refers to polynucleotides such as 25 deoxyribonucleic acid (DNA), and, where appropriate, ribonucleic acid (RNA). The term should also be understood to include, as equivalents, analogs of either RNA or DNA made from nucleotide analogs, and, as applicable to the embodiment being described, single-stranded (such as sense or antisense) and double-stranded polynucleotides.

As used herein, the term "gene" or "recombinant gene" refers to a nucleic acid 30 comprising an open reading frame encoding a *pub* polypeptide of the present invention, including both exon and (optionally) intron sequences. A "recombinant gene" refers to nucleic acid encoding a *pub* polypeptide and comprising *pub*-encoding exon sequences, though it may optionally include intron sequences which are either derived from a 35 chromosomal *pub* gene or from an unrelated chromosomal gene. An exemplary recombinant genes encoding the subject *pub* polypeptides is represented by any of SEQ ID Nos: 1, 3, 5 and/or 7. The term "intron" refers to a DNA sequence present in a given *pub* gene which is not translated into protein and is generally found between exons.

As used herein, the term "transfection" means the introduction of a nucleic acid, e.g., an expression vector, into a recipient cell by nucleic acid-mediated gene transfer. "Transformation", as used herein, refers to a process in which a cell's genotype is changed as a result of the cellular uptake of exogenous DNA or RNA, and, for example, the transformed 5 cell expresses a recombinant form of a *pub* polypeptide of the present invention or where anti-sense expression occurs from the transferred gene, the expression of a naturally- occurring form of the *pub* protein is disrupted.

As used herein, the term "vector" refers to a nucleic acid molecule capable of transporting another nucleic acid to which it has been linked. One type of preferred vector is 10 an episome, i.e., a nucleic acid capable of extra-chromosomal replication. Preferred vectors are those capable of autonomous replication and/ expression of nucleic acids to which they are linked. Vectors capable of directing the expression of genes to which they are operatively linked are referred to herein as "expression vectors". In general, expression vectors of utility in recombinant DNA techniques are often in the form of "plasmids" which 15 refer to circular double stranded DNA loops which, in their vector form are not bound to the chromosome. In the present specification, "plasmid" and "vector" are used interchangeably as the plasmid is the most commonly used form of vector. However, the invention is intended to include such other forms of expression vectors which serve equivalent functions and which become known in the art subsequently hereto.

20 "Transcriptional regulatory sequence" is a generic term used throughout the specification to refer to DNA sequences, such as initiation signals, enhancers, and promoters, which induce or control transcription of protein coding sequences with which they are operably linked. In preferred embodiments, transcription of a recombinant *pub* gene is under the control of a promoter sequence (or other transcriptional regulatory sequence) which 25 controls the expression of the recombinant gene in a cell-type in which expression is intended. It will also be understood that the recombinant gene can be under the control of transcriptional regulatory sequences which are the same or which are different from those sequences which control transcription of the naturally-occurring form of the *pub* protein.

As used herein, the term "tissue-specific promoter" means a DNA sequence that 30 serves as a promoter, i.e., regulates expression of a selected DNA sequence operably linked to the promoter, and which effects expression of the selected DNA sequence in specific cells of a tissue, such as cells of a urogenital origin, e.g. renal cells, or cells of a neural origin, e.g. neuronal cells. The term also covers so-called "leaky" promoters, which regulate expression of a selected DNA primarily in one tissue, but cause expression 35 in other tissues as well.

As used herein, a "transgenic animal" is any animal, preferably a non-human mammal, a bird or an amphibian, in which one or more of the cells of the animal contain heterologous nucleic acid introduced by way of human intervention, such as by transgenic techniques well known in the art. The nucleic acid is introduced into the cell, directly or

indirectly by introduction into a precursor of the cell, by way of deliberate genetic manipulation, such as by microinjection or by infection with a recombinant virus. The term genetic manipulation does not include classical cross-breeding, or *in vitro* fertilization, but rather is directed to the introduction of a recombinant DNA molecule. This molecule may 5 be integrated within a chromosome, or it may be extrachromosomally replicating DNA. In the typical transgenic animals described herein, the transgene causes cells to express a recombinant form of a *pub* protein, e.g. either agonistic or antagonistic forms. However, transgenic animals in which the recombinant *pub* gene is silent are also contemplated, as for example, the FLP or CRE recombinase dependent constructs described below. The 10 "non-human animals" of the invention include vertebrates such as rodents, non-human primates, sheep, dog, cow, chickens, amphibians, reptiles, etc. Preferred non-human animals are selected from the rodent family including rat and mouse, most preferably mouse, though transgenic amphibians, such as members of the *Xenopus* genus, and transgenic chickens can also provide important tools for understanding, for example, embryogenesis and tissue 15 patterning. The term "chimeric animal" is used herein to refer to animals in which the recombinant gene is found, or in which the recombinant is expressed in some but not all cells of the animal. The term "tissue-specific chimeric animal" indicates that the recombinant *pub* gene is present and/or expressed in some tissues but not others.

As used herein, the term "transgene" means a nucleic acid sequence (encoding, e.g., a 20 *pub* polypeptide), which is partly or entirely heterologous, i.e., foreign, to the transgenic animal or cell into which it is introduced, or, is homologous to an endogenous gene of the transgenic animal or cell into which it is introduced, but which is designed to be inserted, or is inserted, into the animal's genome in such a way as to alter the genome of the cell into 25 which it is inserted (e.g., it is inserted at a location which differs from that of the natural gene or its insertion results in a knockout). A transgene can include one or more transcriptional regulatory sequences and any other nucleic acid, such as introns, that may be necessary for optimal expression of a selected nucleic acid.

As is well known, genes for a particular polypeptide may exist in single or multiple 30 copies within the genome of an individual. Such duplicate genes may be identical or may have certain modifications, including nucleotide substitutions, additions or deletions, which all still code for polypeptides having substantially the same activity. The term "DNA sequence encoding a *pub* polypeptide" may thus refer to one or more genes within a particular individual. Moreover, certain differences in nucleotide sequences may exist 35 between individual organisms, which are called alleles. Such allelic differences may or may not result in differences in amino acid sequence of the encoded polypeptide yet still encode a protein with the same biological activity.

"Homology" refers to sequence similarity between two peptides or between two nucleic acid molecules. Homology can be determined by comparing a position in each sequence which may be aligned for purposes of comparison. When a position in the

compared sequence is occupied by the same base or amino acid, then the molecules are homologous at that position. A degree of homology between sequences is a function of the number of matching or homologous positions shared by the sequences.

5 "Cells," "host cells" or "recombinant host cells" are terms used interchangeably herein. It is understood that such terms refer not only to the particular subject cell but to the progeny or potential progeny of such a cell. Because certain modifications may occur in succeeding generations due to either mutation or environmental influences, such progeny may not, in fact, be identical to the parent cell, but are still included within the scope of the term as used herein.

10 A "chimeric protein" or "fusion protein" is a fusion of a first amino acid sequence encoding the subject *pub* polypeptide with a second amino acid sequence defining a domain foreign to and not substantially homologous with any domain of the *pub* polypeptide. A chimeric protein may present a foreign domain which is found (albeit in a different protein) in an organism which also expresses the first protein, or it may be an "interspecies", 15 "intergenic", etc. fusion of protein structures expressed by different kinds of organisms.

20 The term "evolutionarily related to", with respect to nucleic acid sequences encoding *pub*, refers to nucleic acid sequences which have arisen naturally in an organism, including naturally occurring mutants. The term also refers to nucleic acid sequences which, while derived from a naturally occurring *pub* genes, have been altered by mutagenesis, as for example, combinatorial mutagenesis described below, yet still encode polypeptides which have at least one activity of a *pub* protein.

25 The term "isolated" as also used herein with respect to nucleic acids, such as DNA or RNA, refers to molecules separated from other DNAs. or RNAs. respectively, that are present in the natural source of the macromolecule. For example, isolated nucleic acids encoding the subject *pub* polypeptides preferably include no more than 10 kilobases (kb) of nucleic acid sequence which naturally immediately flanks particular *pub* gene in genomic DNA, more preferably no more than 5kb of such naturally occurring flanking sequences, and most preferably less than 1.5kb of such naturally occurring flanking sequence. The term isolated as used herein also refers to a nucleic acid or peptide that is substantially free of 30 cellular material, viral material, or culture medium when produced by recombinant DNA techniques, or chemical precursors or other chemicals when chemically synthesized. Moreover, an "isolated nucleic acid" is meant to include nucleic acid fragments which are not naturally occurring as fragments and would not be found in the natural state.

35 As used herein, a "mitotic activating tyrosine phosphatase" refers to a phosphatase which is involved in one or more aspects of cell-cycle progression, e.g., progression from G₀ to G₁, G₁ to S phase and/or G₂ to M phase.

The term "E3-like complex" refers to a protein complex including a *pub* protein ubiquitin ligase and other associated proteins, which protein complex augments or otherwise facilitates the ubiquitination of a protein. In preferred embodiments, the E3-like complex

includes a *pub* protein which is capable of ubiquitinating the mitotic tyrosine phosphatase cdc25. In other preferred embodiments, the E3-like complex includes a *pub* protein which is capable of ubiquitinating the tumor suppressor protein p53.

5 As used herein "E3-like" or "*pub*-dependent ubiquitination" refers to the conjugation of ubiquitin to a protein by a mechanism which requires a *pub* ligase activity.

The term "substrate protein" or "target protein" refers to a protein, preferably a cellular protein, which can be ubiquitinated by a *pub*-dependent reaction pathway.

10 The term "whole lysate" refers to a cell lysate which has not been manipulated, e.g. either fractionated, depleted or charged, beyond the step of merely lysing the cell to form the lysate. The term whole cell lysate does not, however, include lysates derived from cells which produce recombinant forms of one or more of the proteins required to constitute a ubiquitin-conjugating system for *pub*-dependent ubiquitination of a target protein.

15 The term "charged lysate" refers to cell lysates which have been spiked with exogenous, e.g., purified, semi-purified and/or recombinant, forms of one or more components of a *pub*-dependent ubiquitin-conjugating system, or the target protein thereof. The lysate can be charged after the whole cells have been harvested and lysed, or alternatively, by virtue of the cell from which the lysate is generated expressing a recombinant form of one or more of the conjugating system components.

20 The term "semi-purified cell extract" or, alternatively, "fractionated lysate", as used herein, refers to a cell lysate which has been treated so as to substantially remove at least one component of the whole cell lysate, or to substantially enrich at least one component of the whole cell lysate. "Substantially remove", as used herein, means to remove at least 10%, more preferably at least 50%, and still more preferably at least 80% of the component of the whole cell lysate. "Substantially enrich", as used herein, means to enrich by at least 10%, more preferably by at least 30%, and still more preferably at least about 50%, at least one component of the whole cell lysate compared to another component of the whole cell lysate. The component which is removed or enriched can be a component of a ubiquitin-conjugation pathway, e.g., ubiquitin, a target protein, an E1, an E2, an E3-like complex, a cdc25 phosphatase, and the like, or it can be a component which can interfere with a ubiquitin-binding assay, e.g., a protease.

25 The term "semi-purified cell extract" is also intended to include the lysate from a cell, when the cell has been treated so as to have substantially more, or substantially less, of a given component than a control cell. For example, a cell which has been modified (by, e.g., recombinant DNA techniques) to produce none (or very little) of a component of a ubiquitin-conjugation pathway, will, upon cell lysis, yield a semi-purified cell extract.

30 The term "component of a ubiquitin-conjugation pathway", as used herein, refers to a component which can participate in the ubiquitination of a target protein either *in vivo* or *in vitro*. Exemplary components of a ubiquitin-conjugation pathway include ubiquitin, an E1, an E2, an E3-like complex, a target protein, and the like.

By "semi-purified", with respect to protein preparations, it is meant that the proteins have been previously separated from other cellular or viral proteins. For instance, in contrast to whole cell lysates, the proteins of reconstituted conjugation system, together with the target protein, can be present in the mixture to at least 50% purity relative to all other proteins in 5 the mixture, more preferably are present at at least 75% purity, and even more preferably are present at 90-95% purity.

The term "purified protein", with respect to components of the ubiquitination pathway, refers to a preparation of a protein or proteins which are preferably isolated from, or otherwise substantially free of, other proteins normally associated with the protein(s) in a 10 cell or cell lysate. The term "substantially free of other cellular proteins" (also referred to herein as "contaminating proteins") is defined as encompassing individual preparations of each of the component proteins comprising less than 20% (by dry weight) contaminating protein, and preferably comprises less than 5% contaminating protein. Functional forms of each of the component proteins can be prepared as purified preparations by using a cloned 15 gene as described in the attached examples. By "purified", it is meant, when referring to the component proteins preparations used to generate the reconstituted protein mixture, that the indicated molecule is present in the substantial absence of other biological macromolecules, such as other proteins (particularly other proteins which may substantially mask, diminish, confuse or alter the characteristics of the component proteins either as purified preparations 20 or in their function in the subject reconstituted mixture). The term "purified" as used herein preferably means at least 80% by dry weight, more preferably in the range of 95-99% by weight, and most preferably at least 99.8% by weight, of biological macromolecules of the same type present (but water, buffers, and other small molecules, especially molecules having a molecular weight of less than 5000, can be present). The term "pure" as used herein 25 preferably has the same numerical limits as "purified" immediately above. "Isolated" and "purified" do not encompass either protein in its native state (e.g. as a part of a cell), or as part of a cell lysate, or that have been separated into components (e.g., in an acrylamide gel) but not obtained either as pure (e.g. lacking contaminating proteins) substances or solutions. The term isolated as used herein also refers to a component protein that is substantially free 30 of cellular material or culture medium when produced by recombinant DNA techniques, or chemical precursors or other chemicals when chemically synthesized.

As described below, one aspect of the invention pertains to isolated nucleic acid having a nucleotide sequence encoding a *pub* protein, and/or equivalents of such nucleic acids. The term nucleic acid as used herein is intended to include fragments and equivalents. 35 The term equivalent is understood to include nucleotide sequences encoding functionally equivalent *pub* proteins or functionally equivalent polypeptides which, for example, retain the ability to bind to a mitotic activating tyrosine phosphatase. Equivalent nucleotide sequences will include sequences that differ by one or more nucleotide substitutions, additions or deletions, such as allelic variants; and will, therefore, include sequences that

differ from the nucleotide sequence of the gene encoding h-pub1 shown in SEQ ID No: 1 or the gene encoding s-pub1 shown in SEQ ID No: 3, the h-pub2 sequence shown in SEQ ID No. 5 or the h-pub3 sequence shown in SEQ ID No. 7, due to the degeneracy of the genetic code. Equivalents will also include nucleotide sequences that hybridize under stringent 5 conditions (i.e., equivalent to about 20-27°C below the melting temperature (T_m) of the DNA duplex formed in about 1M salt) to the nucleotide sequence of *pub* gene represented in SEQ ID No: 1, SEQ ID No: 3, SEQ ID No. 5 or SEQ ID No. 7. In one embodiment, equivalents will further include nucleic acid sequences derived from and evolutionarily 10 related to, a nucleotide sequences shown in SEQ ID No: 1. SEQ ID No: 3. SEQ ID No: 5 or SEQ ID No. 7.

Moreover, it will be generally appreciated that, under certain circumstances, it may be advantageous to provide homologs of the subject *pub* proteins, which homologs function in a limited capacity as one of either an agonists (mimetic) or an antagonist in order to promote or inhibit only a subset of the biological activities of the naturally-occurring form of the protein. Thus, specific biological effects can be elicited by treatment with a homolog of 15 limited function, and with fewer side effects relative to treatment with agonists or antagonists which are directed to all of a *pub* protein's biological activities. For instance, antagonistic homologs can be generated which interfere with the ability of the wild-type ("authentic") pub1 protein to associate with cdc25 phosphatase, but which do not substantially interfere with the formation of complexes between pub1 and other cellular 20 proteins, such as may be involved in other regulatory mechanisms of the cell.

Polypeptides referred to herein as *pub* polypeptides preferably have an amino acid sequence corresponding to all or a portion of the pub1 amino acid sequence shown in SEQ ID No. 2 or in SEQ ID No. 4, or the pub2 amino acid sequence shown in SEQ ID No. 6, or the pub3 amino acid sequence shown in SEQ ID No. 8, or are homologous with one of these 25 proteins, such as other human paralogs, or mammalian orthologs. In general, the biological activity of a *pub* polypeptide will be characterized as including the ability to transfer an ubiquitin molecule from the relevant ubiquitin conjugating enzyme (UBC) to a lysine residue of its target through a *pub* ubiquitin thioester intermediate; and an ability to 30 translocate to specific phospholipid membranes in the presence of calcium. The above notwithstanding, the biological activity of a *pub* polypeptide may be characterized by one or more of the following attributes: an ability to regulate the cell-cycle of an eukaryotic cell, especially a mammalian cell (e.g., of a human cell), or a yeast cell such as a *Schizosaccharomyces* cell; an ability to modulate proliferation/cell growth of a eukaryotic 35 cell; an ability to modulate entry of a mammalian or yeast cell into M phase; an ability to ubiquitinate a cell-cycle regulator, e.g. a mitotic activating tyrosine phosphatase, e.g. cdc25. Such activities may be manifested by the ability to control the steady state level of cdc25 phosphatase, and thus to control the degree of dephosphorylation of a cyclin dependent kinase. The *pub* polypeptides of the present invention may also function to modulate

differentiation of cells/tissue. The subject polypeptides of this invention may also be capable of modulating cell growth or proliferation by influencing the action of other cellular proteins. A *pub* polypeptide can be a specific agonist of the function of the wild-type form of the protein, or can be a specific antagonist, such as a catalytically inactive mutant. Other 5 biological activities of the subject *pub* proteins are described herein, or will be reasonably apparent to those skilled in the art in light of the present disclosure.

In one embodiment, the nucleic acid of the invention encodes a polypeptide which is an agonist or antagonist of the naturally occurring h-*pub1* protein and comprises an amino acid sequence identical or homologous to the amino acid sequence represented in SEQ ID 10 No. 2. Preferred nucleic acids encode a polypeptide at least 60% homologous, more preferably 70% homologous and most preferably 80% homologous with an amino acid sequence shown in SEQ ID No. 2. Nucleic acids which encode polypeptides having an activity of a p19 protein and having at least about 90%, more preferably at least about 95%, and most preferably at least about 98-99% homology with a sequence shown in SEQ ID No. 15 2 are also within the scope of the invention. Preferably, the nucleic acid is a cDNA molecule comprising at least a portion of the nucleotide sequence encoding an h-*pub1* protein shown in SEQ ID No. 2. A preferred portion of the cDNA molecule designated by SEQ ID No. 1 includes the coding region of the molecule.

In one embodiment, the nucleic acid of the invention encodes a polypeptide which is an agonist or antagonist of the naturally occurring h-*pub2* protein and comprises an amino 20 acid sequence identical or homologous to the amino acid sequence represented in SEQ ID No. 6. Preferred nucleic acids encode a polypeptide at least 60% homologous, more preferably 70% homologous and most preferably 80% homologous with an amino acid sequence shown in SEQ ID No. 6. Nucleic acids which encode polypeptides having an 25 activity of a *pub2* protein and having at least about 90%, more preferably at least about 95%, and most preferably at least about 98-99% homology with a sequence shown in SEQ ID No. 6 are also within the scope of the invention. Preferably, the nucleic acid is a cDNA molecule comprising at least a portion of the nucleotide sequence encoding an h-*pub2* protein shown 30 in SEQ ID No. 6. A preferred portion of the cDNA molecule designated by SEQ ID No. 5 includes the coding region of the molecule.

In another embodiment, the nucleic acid of the invention encodes a polypeptide which is an agonist or antagonist of the naturally occurring h-*pub3* protein and comprises an amino acid sequence identical or homologous to the amino acid sequence represented in SEQ ID No. 8. Preferred nucleic acids encode a polypeptide at least 60% homologous, more 35 preferably 70% homologous and most preferably 80% homologous with an amino acid sequence shown in SEQ ID No. 8. Nucleic acids which encode polypeptides having an activity of a *pub3* protein and having at least about 90%, more preferably at least about 95%, and most preferably at least about 98-99% homology with a sequence shown in SEQ ID No. 8 are also within the scope of the invention. Preferably, the nucleic acid is a cDNA molecule

comprising at least a portion of the nucleotide sequence encoding an h-pub2 protein shown in SEQ ID No. 8. A preferred portion of the cDNA molecule designated by SEQ ID No. 7 includes the coding region of the molecule.

In another embodiment, the nucleic acid of the invention encodes a polypeptide which is an agonist or antagonist of the naturally occurring s-pub1 protein and comprises an amino acid sequence identical or homologous to the amino acid sequence represented in SEQ ID No. 4. Preferred nucleic acids encode a polypeptide at least 60% homologous, more preferably 70% homologous and most preferably 80% homologous with an amino acid sequence shown in SEQ ID No. 4. Nucleic acids which encode polypeptides having an activity of an s-pub1 protein and having at least about 90%, more preferably at least about 95%, and most preferably at least about 98-99% homology with a sequence shown in SEQ ID No. 4 are also within the scope of the invention. Preferably, the nucleic acid is a cDNA molecule comprising at least a portion of the nucleotide sequence encoding an s-pub1 protein shown in SEQ ID No. 4. A preferred portion of the cDNA molecule shown in SEQ ID No. 3 includes the coding region of the molecule.

Isolated nucleic acids which differ from the nucleotide sequences shown in SEQ ID No: 1, SEQ ID No: 3, SEQ ID No: 5 or SEQ ID No. 7 due to degeneracy in the genetic code are also within the scope of the invention. For example, a number of amino acids are designated by more than one triplet. Codons that specify the same amino acid, or synonyms (for example, CAU and CAC are synonyms for histidine) may result in "silent" mutations which do not affect the amino acid sequence of the protein. However, it is expected that DNA sequence polymorphisms that do lead to changes in the amino acid sequences of the subject *pub* proteins will exist among mammalian cells. One skilled in the art will appreciate that these variations in one or more nucleotides (up to about 3-4% of the nucleotides) of the nucleic acids encoding a particular *pub* protein may exist among individuals of a given species due to natural allelic variation. Any and all such nucleotide variations and resulting amino acid polymorphisms are within the scope of this invention.

Fragments of the nucleic acid encoding a biologically active portion of the subject *pub* proteins are also within the scope of the invention. As used herein, a fragment of the nucleic acid encoding an active portion of a *pub* protein refers to a nucleotide sequence having fewer nucleotides than the nucleotide sequence encoding the full length amino acid sequence of, for example, the *pub* protein represented in SEQ ID Nos: 2, 4, 6 and/or 8, and which encodes a polypeptide which retains at least a portion of the biological activity of the full-length protein as defined herein, or alternatively, which is functional as an antagonist of the biological activity of the full-length protein. For example, such fragments include, as appropriate to the full-length protein from which they are derived, a polypeptide containing a CaLB domain and capable of associating with a phospholipid membrane in a calcium dependent manner, an ATP binding motif, and/or a catalytically active domain, e.g., a hect domain.

Nucleic acids within the scope of the invention may also contain linker sequences, modified restriction endonuclease sites and other sequences useful for molecular cloning, expression or purification of such recombinant polypeptides.

As indicated by the examples set out below, a nucleic acid encoding a *pub* polypeptide may be obtained from mRNA or genomic DNA present in any of a number of 5 mammalian cells in accordance with protocols described herein, as well as those generally known to those skilled in the art. A cDNA encoding a *pub* polypeptide, for example, can be obtained by isolating total mRNA from a cell, e.g. a mammalian cell, e.g. a human cell. Double stranded cDNAs can then be prepared from the total mRNA, and subsequently 10 inserted into a suitable plasmid or bacteriophage vector using any one of a number of known techniques. A gene encoding a *pub* protein can also be cloned using established polymerase chain reaction techniques in accordance with the nucleotide sequence information provided 15 by the invention.

Another aspect of the invention relates to the use of the isolated nucleic acid in 20 "antisense" therapy. As used herein, antisense therapy refers to administration or *in situ* generation of oligonucleotide probes or their derivatives which specifically hybridize (e.g. binds) under cellular conditions with the cellular mRNA and/or genomic DNA encoding one 25 of the subject *pub* proteins so as to inhibit expression of that protein, e.g. by inhibiting transcription and/or translation. The binding may be by conventional base pair complementarity, or, for example, in the case of binding to DNA duplexes, through specific 30 interactions in the major groove of the double helix. In general, antisense therapy refers to the range of techniques generally employed in the art, and includes any therapy which relies on specific binding to oligonucleotide sequences.

An antisense construct of the present invention can be delivered, for example, as an 35 expression plasmid which, when transcribed in the cell, produces RNA which is complementary to at least a unique portion of the cellular mRNA which encodes a *pub* protein. Alternatively, the antisense construct is an oligonucleotide probe which is generated *ex vivo* and which, when introduced into the cell causes inhibition of expression by hybridizing with the mRNA and/or genomic sequences encoding a *pub* protein. Such oligonucleotide probes are preferably modified oligonucleotide which are resistant to endogenous nucleases, e.g. exonucleases and/or endonucleases, and is therefore stable *in vivo*. Exemplary nucleic acid molecules for use as antisense oligonucleotides are phosphoramidate, phosphothioate and methylphosphonate analogs of DNA (see also U.S. Patents 5,176,996; 5,264,564; and 5,256,775). Additionally, general approaches to 40 constructing oligomers useful in antisense therapy have been reviewed, for example, by van der Krol et al., (1988) *Biotechniques* 6:958-976; and Stein et al., (1988) *Cancer Res* 48:2659-2668.

Accordingly, the modified oligomers of the invention are useful in therapeutic, diagnostic, and research contexts. In therapeutic applications, the oligomers are utilized in a

manner appropriate for antisense therapy in general. For such therapy, the oligomers of the invention can be formulated for a variety of modes of administration, including systemic and topical or localized administration. Techniques and formulations generally may be found in *Remmington's Pharmaceutical Sciences*, Meade Publishing Co., Easton, PA. For systemic 5 administration, injection is preferred, including intramuscular, intravenous, intraperitoneal, and subcutaneous for injection, the oligomers of the invention can be formulated in liquid solutions, preferably in physiologically compatible buffers such as Hank's solution or Ringer's solution. In addition, the oligomers may be formulated in solid form and redissolved or suspended immediately prior to use. Lyophilized forms are also included.

10 Systemic administration can also be by transmucosal or transdermal means, or the compounds can be administered orally. For transmucosal or transdermal administration, penetrants appropriate to the barrier to be permeated are used in the formulation. Such penetrants are generally known in the art, and include, for example, for transmucosal administration bile salts and fusidic acid derivatives. In addition, detergents may be used to 15 facilitate permeation. Transmucosal administration may be through nasal sprays or using suppositories. For oral administration, the oligomers are formulated into conventional oral administration forms such as capsules, tablets, and tonics. For topical administration, the oligomers of the invention are formulated into ointments, salves, gels, or creams as generally known in the art.

20 In addition to use in therapy, the oligomers of the invention may be used as diagnostic reagents to detect the presence or absence of the target DNA or RNA sequences to which they specifically bind.

25 In another aspect of the invention, the subject nucleic acid is provided in an expression vector comprising a nucleotide sequence encoding a subject *pub* polypeptide and operably linked to at least one regulatory sequence. Operably linked is intended to mean that the nucleotide sequence is linked to a regulatory sequence in a manner which allows expression of the nucleotide sequence. Regulatory sequences are art-recognized and are selected to direct expression of the polypeptide having an activity of a *pub* protein. Accordingly, the term regulatory sequence includes promoters, enhancers and other 30 expression control elements. Exemplary regulatory sequences are described in Goeddel; *Gene Expression Technology: Methods in Enzymology*. Academic Press, San Diego, CA (1990). For instance, any of a wide variety of expression control sequences that control the expression of a DNA sequence when operatively linked to it may be used in these vectors to express DNA sequences encoding the *pub* proteins of this invention. Such useful expression 35 control sequences, include, for example, the early and late promoters of SV40, adenovirus or cytomegalovirus immediate early promoter, the lac system, the trp system, the TAC or TRC system, T7 promoter whose expression is directed by T7 RNA polymerase, the major operator and promoter regions of phage lambda, the control regions for fd coat protein, the promoter for 3-phosphoglycerate kinase or other glycolytic enzymes, the promoters of acid

phosphatase, e.g., Pho5, the promoters of the yeast α -mating factors, the polyhedron promoter of the baculovirus system and other sequences known to control the expression of genes of prokaryotic or eukaryotic cells or their viruses, and various combinations thereof. It should be understood that the design of the expression vector may depend on such factors as 5 the choice of the host cell to be transformed and/or the type of protein desired to be expressed. Moreover, the vector's copy number, the ability to control that copy number and the expression of any other proteins encoded by the vector, such as antibiotic markers, should also be considered.

As will be apparent, the subject gene constructs can be used to cause expression of 10 the subject *pub* polypeptides in cells propagated in culture, e.g. to produce proteins or polypeptides, including fusion proteins or polypeptides, for purification.

In addition, recombinant expression of the subject *pub* polypeptides in cultured cells can be useful for controlling differentiation states of cells *in vitro*, for instance, by controlling the steady state level of activation of cdc25 and thus, the activation of a CDK, 15 e.g., cdc2, or by controlling the half-life of the tumor suppressor p53. To illustrate, *in vitro* neuronal culture systems have proved to be fundamental and indispensable tools for the study of neural development, as well as the identification of neurotrophic factors. Once a neuronal cell has become terminally-differentiated, it typically will not change to another terminally differentiated cell-type. However, neuronal cells can nevertheless sometimes lose 20 their differentiated state. This is commonly observed when they are grown in culture from adult tissue, and when they form a blastema during regeneration. By preventing the activation of an M-phase CDK, certain of the *pub* homologs (presumably agonist forms) can prevent mitotic progression and hence provide a means for ensuring an adequately restrictive environment in order to maintain neuronal cells at various stages of differentiation, and can 25 be employed, for instance, in cell cultures designed to test the specific activities of trophic factors. Other tissue culture systems which require maintenance of differentiation will be readily apparent to those skilled in the art. In this respect, each of the agonist and antagonist of *pub* activation can be used for *ex vivo* tissue generation, as for example, to enhance the generation of prosthetic tissue devices for implantation.

30 To further illustrate, hyper-proliferative cells can be created by antagonizing the activity of the wild-type *pub* protein, such as by expression of antagonistic homologs, e.g. dominant negative mutants, antisense constructs, or treatment with agents able to disrupt binding of a *pub* protein with, for example, a cdc25 phosphatase. *Pub* antagonists provides a method of transforming mammalian cells to be used as *in vivo* systems to characterize 35 mitotic inhibitors. Conversely, a hypo-proliferative cell can be created by potentiating the activity of the wild type *pub* protein by expression of agonist homologs or treatment with agents that enhance the binding of *pub* to cdc25, and thus reduce the level of cdc25 present in a cell.

Moreover, antagonizing the activity of the wild-type *pub* proteins, such as by expression of antagonistic homologs, antisense constructs, or treatment with agents able to disrupt binding of *pub* proteins with the cdc25 and/or p53 proteins, can be utilized in diagnostic assays to determine if a cell's growth is no longer dependent on the regulatory function of cdc25 or p53 and *pub* proteins, e.g. in determining the phenotype of a transformed cell. To illustrate, a sample of cells from the tissue can be obtained from a patient and dispersed in appropriate cell culture media, a portion of the cells in the sample can be caused to express a recombinant *pub* protein, e.g. by transfection with an expression vector, and subsequent growth of the cells assessed. The ability of cells to proliferate despite expression of an agonistic *pub* protein is indicative of a lack of dependence on cell regulatory pathways which include the *pub* protein, e.g. a cdc25/cdk-dependent pathway(s). Depending on the nature of the tissue of interest, the sample can be in the form of cells isolated from, for example, a blood sample, an exfoliated cell sample, a fine needle aspirant sample, or a biopsied tissue sample. Where the initial sample is a solid mass, the tissue sample can be minced or otherwise dispersed so that cells can be cultured, as is known in the art. Such knowledge can have both prognostic and therapeutic benefits.

Thus, another aspect of the present invention concerns recombinant *pub* proteins which have at least one biological activity of a naturally occurring *pub* protein, or which are naturally occurring mutants thereof. The term "recombinant protein" refers to a protein of the present invention which is produced by recombinant DNA techniques, wherein generally DNA encoding the *pub* protein is inserted into a suitable expression vector which is in turn used to transform a host cell to produce the heterologous protein. Moreover, the phrase "derived from", with respect to a recombinant gene encoding the recombinant *pub* protein, is meant to include within the meaning of "recombinant protein" those proteins having an amino acid sequence of a native *pub* protein, or an amino acid sequence similar thereto which is generated by mutations including substitutions and deletions of a naturally occurring *pub* protein. To illustrate, recombinant proteins preferred by the present invention, in addition to native *pub* proteins, are those recombinantly produced proteins which are at least 60% homologous, more preferably 70% homologous and most preferably 80% homologous with an amino acid sequence shown in SEQ ID No. 2, SEQ ID No. 4, SEQ ID No: 6 and/or SEQ ID No. 8. Polypeptides having an activity of a *pub* protein, such as the ability to transfer an ubiquitin molecule from the relevant ubiquitin conjugating enzyme (UBC) or E2 to a lysine residue of its target through a *pub* ubiquitin thioester intermediate, and having at least about 90%, more preferably at least about 95%, and most preferably at least about 98-99% homology with a sequence shown in SEQ ID No. 2, SEQ ID No. 4, SEQ ID No: 6 and/or SEQ ID No. 8 are also within the scope of the invention. Thus, the present invention pertains to recombinant *pub* proteins which are encoded by genes derived from an eukaryotic cell and which have amino acid sequences evolutionarily related to a *pub* protein represented by one of SEQ ID No. 2, SEQ ID No. 4, SEQ ID No: 6 and/or SEQ ID No. 8.

wherein "evolutionarily related to", refers to *pub* proteins having amino acid sequences which have arisen naturally (e.g. by allelic variance or by differential splicing), as well as mutational variants of *pub* proteins which are derived, for example, by combinatorial mutagenesis.

5 This invention also pertains to a host cell transfected with a recombinant *pub* gene in order to express a polypeptide having an activity of a *pub* protein. The host cell may be any prokaryotic or eukaryotic cell. For example, a *pub* protein of the present invention may be expressed in bacterial cells such as *E. coli*, insect cells (baculovirus), yeast, or mammalian cells. Other suitable host cells are known to those skilled in the art.

10 Accordingly, the present invention further pertains to methods of producing the subject *pub* proteins. For example, a host cell transfected with an expression vector encoding a *pub* polypeptide can be cultured under appropriate conditions to allow expression of the polypeptide to occur. The polypeptide may be secreted and isolated from a mixture of cells and medium containing the polypeptide. Alternatively, the polypeptide may be 15 retained cytoplasmically and the cells harvested, lysed and the protein isolated. A cell culture includes host cells, media and other byproducts. Suitable media for cell culture are well known in the art. The polypeptide can be isolated from cell culture medium, host cells, or both using techniques known in the art for purifying proteins, including ion-exchange chromatography, gel filtration chromatography, ultrafiltration, electrophoresis, and 20 immunoaffinity purification with antibodies specific for particular epitopes of the *pub* protein. In a preferred embodiment, the *pub* protein is a fusion protein containing a domain which facilitates its purification, such as a *pub*-GST fusion protein.

25 Thus, a nucleotide sequence derived from the cloning of the *pub* proteins described in the present invention, encoding all or a selected portion of the protein, can be used to produce a recombinant form of the protein via microbial or eukaryotic cellular processes. Ligating the polynucleotide sequence into a gene construct, such as an expression vector, and transforming or transfecting into hosts, either eukaryotic (yeast, avian, insect or mammalian) or prokaryotic (bacterial cells), are standard procedures used in producing other 30 well-known cell-cycle regulatory proteins, e.g. p53, cyclins, RB, p16, ubc4, E6-AP, and the like. Similar procedures, or modifications thereof, can be employed to prepare recombinant *pub* proteins, or portions thereof, by microbial means or tissue-culture technology in accord with the subject invention.

35 The recombinant *pub* protein can be produced by ligating the cloned gene, or a portion thereof, into a vector suitable for expression in either prokaryotic cells, eukaryotic cells, or both. Expression vehicles for production of a recombinant *pub* protein include plasmids and other vectors. For instance, suitable vectors for the expression of *pub* include plasmids of the types: pBR322-derived plasmids, pEMBL-derived plasmids, pEX-derived plasmids, pBTac-derived plasmids and pUC-derived plasmids for expression in prokaryotic cells, such as *E. coli*.

A number of vectors exist for the expression of recombinant proteins in yeast. For instance, YEP24, YIP5, YEP51, YEP52, pYES2, and YRP17 are cloning and expression vehicles useful in the introduction of genetic constructs into *S. cerevisiae* (see, for example, Broach et al., (1983) in *Experimental Manipulation of Gene Expression*, ed. M. Inouye 5 Academic Press, p. 83, incorporated by reference herein). These vectors can replicate in *E. coli* due the presence of the pBR322 ori, and in *S. cerevisiae* due to the replication determinant of the yeast 2 micron plasmid. In addition, drug resistance markers such as ampicillin can be used.

The preferred mammalian expression vectors contain both prokaryotic sequences to 10 facilitate the propagation of the vector in bacteria, and one or more eukaryotic transcription units that are expressed in eukaryotic cells. The pcDNA1/amp, pcDNA1/neo, pRc/CMV, pSV2gpt, pSV2neo, pSV2-dhfr, pTk2, pRSVneo, pMSG, pSVT7, pko-neo and pHg derived vectors are examples of mammalian expression vectors suitable for transfection of 15 eukaryotic cells. Some of these vectors are modified with sequences from bacterial plasmids, such as pBR322, to facilitate replication and drug resistance selection in both prokaryotic and eukaryotic cells. Alternatively, derivatives of viruses such as the bovine papilloma virus (BPV-1), or Epstein-Barr virus (pHEBo, pREP-derived and p205) can be 20 used for transient expression of proteins in eukaryotic cells. Examples of other viral (including retroviral) expression systems can be found below in the description of gene therapy delivery systems. The various methods employed in the preparation of the plasmids and transformation of host organisms are well known in the art. For other suitable 25 expression systems for both prokaryotic and eukaryotic cells, as well as general recombinant procedures, see *Molecular Cloning A Laboratory Manual*, 2nd Ed., ed. by Sambrook, Fritsch and Maniatis (Cold Spring Harbor Laboratory Press, 1989) Chapters 16 and 17. In some instances, it may be desirable to express the recombinant *pub* protein by the use of a baculovirus expression system. Examples of such baculovirus expression systems include 30 pVL-derived vectors (such as pVL1392, pVL1393 and pVL941), pAcUW-derived vectors (such as pAcUW1), and pBlueBac-derived vectors (such as the β -gal containing pBlueBac III).

When expression of a carboxy terminal fragment of the full-length *pub* proteins is 35 desired, i.e. a truncation mutant, it may be necessary to add a start codon (ATG) to the oligonucleotide fragment containing the desired sequence to be expressed. It is well known in the art that a methionine at the N-terminal position can be enzymatically cleaved by the use of the enzyme methionine aminopeptidase (MAP). MAP has been cloned from *E. coli* (Ben-Bassat et al., (1987) *J. Bacteriol.* 169:751-757) and *Salmonella typhimurium* and its *in vitro* activity has been demonstrated on recombinant proteins (Miller et al., (1987) *Proc. Natl. Acad. Sci. USA* 84:2718-1722). Therefore, removal of an N-terminal methionine, if desired, can be achieved either *in vivo* by expressing such recombinant polypeptides in a

host which produces MAP (e.g., *E. coli* or CM89 or *S. cerevisiae*), or *in vitro* by use of purified MAP (e.g., procedure of Miller et al.).

Alternatively, the coding sequences for the polypeptide can be incorporated as a part of a fusion gene including a nucleotide sequence encoding a different polypeptide. This type of expression system can be useful under conditions where it is desirable to produce an immunogenic fragment of the *pub* protein. For example, the VP6 capsid protein of rotavirus can be used as an immunologic carrier protein for portions of polypeptide, either in the monomeric form or in the form of a viral particle. The nucleic acid sequences corresponding to the portion of the *pub* protein to which antibodies are to be raised can be incorporated into a fusion gene construct which includes coding sequences for a late vaccinia virus structural protein to produce a set of recombinant viruses expressing fusion proteins comprising a portion of the protein as part of the virion. The Hepatitis B surface antigen can also be utilized in this role as well. Similarly, chimeric constructs coding for fusion proteins containing a portion of a *pub* protein and the poliovirus capsid protein can be created to enhance immunogenicity (see, for example, EP Publication No. 0259149; and Evans et al., (1989) *Nature* 339:385; Huang et al., (1988) *J. Virol.* 62:3855; and Schlienger et al., (1992) *J. Virol.* 66:2).

The Multiple Antigen Peptide system for peptide-based immunization can be utilized, wherein a desired portion of a *pub* protein is obtained directly from organo-chemical synthesis of the peptide onto an oligomeric branching lysine core (see, for example, Posnett et al., (1988) *JBC* 263:1719 and Nardelli et al., (1992) *J. Immunol.* 148:914). Antigenic determinants of the *pub* protein can also be expressed and presented by bacterial cells.

In addition to utilizing fusion proteins to enhance immunogenicity, it is widely appreciated that fusion proteins can also facilitate the expression of proteins. For example, the *pub* protein of the present invention can be generated as a glutathione-S-transferase (GST) fusion proteins. Such GST fusion proteins can be used to simply purification of the *pub* protein, such as through the use of glutathione-derivatized matrices (see, for example, *Current Protocols in Molecular Biology*, eds. Ausubel et al., (N.Y.: John Wiley & Sons, 1991)).

In another embodiment, a fusion gene coding for a purification leader sequence, such as a poly-(His)/enterokinase cleavage site sequence at the N-terminus of the desired portion of the recombinant protein, can allow purification of the expressed fusion protein by affinity chromatography using a Ni²⁺ metal resin. The purification leader sequence can then be subsequently removed by treatment with enterokinase to provide the purified *pub* protein (e.g., see Hochuli et al., (1987) *J. Chromatography* 411:177; and Janknecht et al., *Proc. Natl. Acad. Sci. USA* 88:8972).

Techniques for making fusion genes are well known. Essentially, the joining of various DNA fragments coding for different polypeptide sequences is performed in

accordance with conventional techniques, employing blunt-ended or stagger-ended termini for ligation, restriction enzyme digestion to provide for appropriate termini, filling-in of cohesive ends as appropriate, alkaline phosphatase treatment to avoid undesirable joining, and enzymatic ligation. In another embodiment, the fusion gene can be synthesized by conventional techniques including automated DNA synthesizers. Alternatively, PCR amplification of gene fragments can be carried out using anchor primers which give rise to complementary overhangs between two consecutive gene fragments which can subsequently be annealed to generate a chimeric gene sequence (see, for example, *Current Protocols in Molecular Biology*, eds. Ausubel et al., John Wiley & Sons: 1992).

The present invention also makes available isolated and/or purified forms of the subject *pub* polypeptides, which are isolated from, or otherwise substantially free of other intracellular proteins, especially cell-cycle regulatory proteins, e.g. p53, cdc25 and/or E2 enzymes, which might normally be associated with the *pub* protein. The term "substantially free of other cellular proteins" (also referred to herein as "contaminating proteins") is defined as encompassing, for example, *pub* preparations comprising less than 20% (by dry weight) contaminating protein, and preferably comprises less than 5% contaminating protein. Functional forms of the *pub* polypeptide can be prepared, for the first time, as purified preparations by using a cloned gene as described herein. By "purified", it is meant, when referring to a polypeptide, that the indicated molecule is present in the substantial absence of other biological macromolecules, such as other proteins (particularly other cell-cycle proteins such as p53 and/or cdc25 phosphatase, as well as other contaminating proteins). The term "purified" as used herein preferably means at least 80% by dry weight, more preferably in the range of 95-99% by weight, and most preferably at least 99.8% by weight, of biological macromolecules of the same type present (but water, buffers, and other small molecules, especially molecules having a molecular weight of less than 5000, can be present). The term "pure" as used herein preferably has the same numerical limits as "purified" immediately above. "Isolated" and "purified" do not encompass either natural materials in their native state or natural materials that have been separated into components (e.g., in an acrylamide gel) but not obtained either as pure (e.g. lacking contaminating proteins, or chromatography reagents such as denaturing agents and polymers, e.g. acrylamide or agarose) substances or solutions.

The subject polypeptides can also be provided in pharmaceutically acceptable carriers for formulated for a variety of modes of administration, including systemic and topical or localized administration. Techniques and formulations generally may be found in *Remmington's Pharmaceutical Sciences*, Meade Publishing Co., Easton, PA. In an exemplary embodiment, the *pub* polypeptide is provided for transmucosal or transdermal delivery. For such administration, penetrants appropriate to the barrier to be permeated are used in the formulation with the polypeptide. Such penetrants are generally known in the art, and include, for example, for transmucosal administration bile salts and fusidic acid

derivatives. In addition, detergents may be used to facilitate permeation. Transmucosal administration may be through nasal sprays or using suppositories. For topical administration, the oligomers of the invention are formulated into ointments, salves, gels, or creams as generally known in the art.

5 Another aspect of the invention relates to polypeptides derived from the full-length *pub* protein. Isolated peptidyl portions of the subject *pub* protein can be obtained by screening polypeptides recombinantly produced from the corresponding fragment of the nucleic acid encoding such polypeptides. In addition, fragments can be chemically synthesized using techniques known in the art such as conventional Merrifield solid phase f-
10 Moc or t-Boc chemistry. For example, *pub* protein can be arbitrarily divided into fragments of desired length with no overlap of the fragments, or preferably divided into overlapping fragments of a desired length. The fragments can be produced (recombinantly or by chemical synthesis) and tested to identify those peptidyl fragments which can function as either agonists or antagonists of, for example, cdc25 degradation, such as by microinjection
15 assays. In an illustrative embodiment, peptidyl portions of *pub* protein can be tested for cdc25-binding activity, as well as inhibitory ability, by expression as, for example, thioredoxin fusion proteins, each of which contains a discrete fragment of the *pub* protein (see, for example, U.S. Patents 5,270,181 and 5,292,646; and PCT publication WO94/02502).

20 It is also possible to modify the structure of the subject *pub* proteins for such purposes as enhancing therapeutic or prophylactic efficacy, or stability (e.g., *ex vivo* shelf life and resistance to proteolytic degradation *in vivo*). Such modified polypeptides, when designed to retain at least one activity of the naturally-occurring form of the protein, are considered functional equivalents of the *pub* polypeptides described in more detail herein. Such modified polypeptides can be produced, for instance, by amino acid substitution,
25 deletion, or addition.

20 For instance, it is reasonable to expect, for example, that an isolated replacement of a leucine with an isoleucine or valine, an aspartate with a glutamate, a threonine with a serine, or a similar replacement of an amino acid with a structurally related amino acid (i.e. conservative mutations) will not have a major effect on the biological activity of the resulting molecule. Conservative replacements are those that take place within a family of amino acids that are related in their side chains. Genetically encoded amino acids can be divided into four families: (1) acidic = aspartate, glutamate; (2) basic = lysine, arginine, histidine; (3) nonpolar = alanine, valine, leucine, isoleucine, proline, phenylalanine, methionine, tryptophan; and (4) uncharged polar = glycine, asparagine, glutamine, cysteine, serine, threonine, tyrosine. Phenylalanine, tryptophan, and tyrosine are sometimes classified jointly as aromatic amino acids. In similar fashion, the amino acid repertoire can be grouped as (1) acidic = aspartate, glutamate; (2) basic = lysine, arginine, histidine, (3) aliphatic = glycine, alanine, valine, leucine, isoleucine, serine, threonine, with serine and threonine optionally be grouped separately as aliphatic-hydroxyl; (4) aromatic = phenylalanine,

tyrosine, tryptophan; (5) amide = asparagine, glutamine; and (6) sulfur-containing = cysteine and methionine. (see, for example, *Biochemistry*, 2nd ed., Ed. by L. Stryer, W.H. Freeman and Co., 1981). Whether a change in the amino acid sequence of a polypeptide results in a functional homolog can be readily determined by assessing the ability of the variant polypeptide to produce a response in cells in a fashion similar to the wild-type protein. For instance, such variant forms of *pub* can be assessed for their ability to bind to a cdc25 phosphatase of the present invention or other cellular protein. Polypeptides in which more than one replacement has taken place can readily be tested in the same manner.

This invention further contemplates a method of generating sets of combinatorial mutants of the subject *pub* proteins, as well as truncation mutants, and is especially useful for identifying potential variant sequences (e.g. homologs) that are functional in binding to a regulatory protein, especially cdc25 phosphatase. The purpose of screening such combinatorial libraries is to generate, for example, *pub* homologs which can act as either agonists or antagonist, or alternatively, which possess novel activities all together. To illustrate, homologs can be engineered by the present method to provide more efficient binding to cdc25 phosphatase, yet have a significantly reduced binding affinity for other cell-cycle regulatory proteins relative to the naturally-occurring form of the protein. Thus, combinatorially-derived homologs can be generated which have a selective potency relative to a naturally occurring *pub* protein. Such proteins, when expressed from recombinant DNA constructs, can be used in gene therapy protocols.

Likewise, mutagenesis can give rise to homologs which have intracellular half-lives dramatically different than the corresponding wild-type protein. For example, the altered protein can be rendered either more stable or less stable to proteolytic degradation or other cellular process which result in destruction of, or otherwise inactivation of the *pub* protein. Such homologs, and the genes which encode them, can be utilized to alter the envelope of *pub* expression by modulating the half-life of the protein. For instance, a short half-life can give rise to more transient biological effects and, when part of an inducible expression system, can allow tighter control of recombinant *pub* protein levels within the cell. As above, such proteins, and particularly their recombinant nucleic acid constructs, can be used in gene therapy protocols.

In similar fashion, *pub* homologs can be generated by the present combinatorial approach to act as antagonists, in that they are able to interfere with the ability of the corresponding wild-type protein to regulate cell proliferation.

In a representative embodiment of this method, the amino acid sequences for a population of *pub* protein homologs are aligned, preferably to promote the highest homology possible. Such a population of variants can include, for example, homologs from one or more species, or homologs from the same species but which differ due to mutation. Amino acids which appear at each position of the aligned sequences are selected to create a degenerate set of combinatorial sequences. In a preferred embodiment, the combinatorial

library is produced by way of a degenerate library of genes encoding a library of polypeptides which each include at least a portion of potential *pub* protein sequences. For instance, a mixture of synthetic oligonucleotides can be enzymatically ligated into gene sequences such that the degenerate set of potential *pub* nucleotide sequences are expressible as individual polypeptides, or alternatively, as a set of larger fusion proteins (e.g. for phage display).

There are many ways by which the library of potential homologs can be generated from a degenerate oligonucleotide sequence. Chemical synthesis of a degenerate gene sequence can be carried out in an automatic DNA synthesizer, and the synthetic genes then be ligated into an appropriate gene for expression. The purpose of a degenerate set of genes is to provide, in one mixture, all of the sequences encoding the desired set of potential *pub* sequences. The synthesis of degenerate oligonucleotides is well known in the art (see for example, Narang, SA (1983) *Tetrahedron* 39:3; Itakura et al. (1981) *Recombinant DNA, Proc. 3rd Cleveland Sympos. Macromolecules.* ed. AG Walton, Amsterdam: Elsevier pp273-289; Itakura et al., (1984) *Annu. Rev. Biochem.* 53:323; Itakura et al., (1984) *Science* 198:1056; Ike et al., (1983) *Nucleic Acid Res.* 11:477. Such techniques have been employed in the directed evolution of other proteins (see, for example, Scott et al., (1990) *Science* 249:386-390; Roberts et al., (1992) *Proc. Natl. Acad. Sci. USA* 89:2429-2433; Devlin et al., (1990) *Science* 249: 404-406; Cwirla et al., (1990) *Proc. Natl. Acad. Sci. USA* 87: 6378-6382; as well as U.S. Patent Nos: 5,223,409, 5,198,346, and 5,096,815).

Alternatively, other forms of mutagenesis can be utilized to generate a combinatorial library. For example, *pub* homologs (both agonist and antagonist forms) can be generated and isolated from a library by screening using, for example, alanine scanning mutagenesis and the like (Ruf et al., (1994) *Biochemistry* 33:1565-1572; Wang et al., (1994) *J. Biol. Chem.* 269:3095-3099; Balint et al., (1993) *Gene* 137:109-118; Grodberg et al., (1993) *Eur. J. Biochem.* 218:597-601; Nagashima et al., (1993) *J. Biol. Chem.* 268:2888-2892; Lowman et al., (1991) *Biochemistry* 30:10832-10838; and Cunningham et al., (1989) *Science* 244:1081-1085), by linker scanning mutagenesis (Gustin et al., (1993) *Virology* 193:653-660; Brown et al., (1992) *Mol. Cell Biol.* 12:2644-2652; McKnight et al., (1982) *Science* 232:316); by saturation mutagenesis (Meyers et al., (1986) *Science* 232:613); by PCR mutagenesis (Leung et al., (1989) *Method Cell Mol Biol* 1:11-19); or by random mutagenesis (Miller et al., (1992) *A Short Course in Bacterial Genetics*, CSHL Press, Cold Spring Harbor, NY; and Greener et al., (1994) *Strategies in Mol Biol* 7:32-34). Linker scanning mutagenesis, particularly in a combinatorial setting, is an attractive method for identifying truncated (bioactive) forms of the *pub* proteins.

A wide range of techniques are known in the art for screening gene products of combinatorial libraries made by point mutations and truncations, and, for that matter, for screening cDNA libraries for gene products having a certain property. Such techniques will be generally adaptable for rapid screening of the gene libraries generated by the

combinatorial mutagenesis of *pub* homologs. The most widely used techniques for screening large gene libraries typically comprises cloning the gene library into replicable expression vectors, transforming appropriate cells with the resulting library of vectors, and expressing the combinatorial genes under conditions in which detection of a desired activity 5 facilitates relatively easy isolation of the vector encoding the gene whose product was detected. Each of the illustrative assays described below are amenable to high through-put analysis as necessary to screen large numbers of degenerate sequences created by combinatorial mutagenesis techniques.

In an illustrative embodiment of a screening assay, candidate *pub* combinatorial gene 10 products, are displayed on the surface of a cell, and the ability of particular cells or viral particles to bind a cdc25 polypeptide sequence, or other binding partners (e.g., p53) of *pub* via this gene product is detected in a "panning assay". For instance, the *pub* gene library can be cloned into the gene for a surface membrane protein of a bacterial cell (Ladner et al., WO 88/06630; Fuchs et al., (1991) *Bio/Technology* 9:1370-1371; and Goward et al., (1992) *TIBS* 15 18:136-140), and the resulting fusion protein detected by panning, e.g. using a fluorescently labeled molecule which binds the *pub* protein, e.g. FITC-cdc25, to score for potentially functional homologs. Cells can be visually inspected and separated under a fluorescence-activated cell sorter. While the preceding description is directed to embodiments exploiting 20 the interaction between *pub* and a cdc25 polypeptide, it will be understood that similar embodiments can be generated using, for example, a *pub* polypeptide displayed on the surface of a cell and examining the ability of those *pub*-expressing cells to bind other binding partners of *pub*.

In similar fashion, the gene library can be expressed as a fusion protein on the surface 25 of a viral particle. For instance, in the filamentous phage system, foreign peptide sequences can be expressed on the surface of infectious phage, thereby conferring two significant benefits. First, since these phage can be applied to affinity matrices at very high concentrations, a large number of phage can be screened at one time. Second, since each infectious phage displays the combinatorial gene product on its surface, if a particular phage 30 is recovered from an affinity matrix in low yield, the phage can be amplified by another round of infection. The group of almost identical *E. coli* filamentous phages M13, fd, and f1 are most often used in phage display libraries, as either of the phage gIII or gVIII coat proteins can be used to generate fusion proteins without disrupting the ultimate packaging of the viral particle (Ladner et al., PCT publication WO 90/02909; Garrard et al., PCT 35 publication WO 92/09690; Marks et al., (1992) *J. Biol. Chem.* 267:16007-16010; Griffiths et al., (1993) *EMBO J.* 12:725-734; Clackson et al., (1991) *Nature* 352:624-628; and Barbas et al., (1992) *Proc. Natl. Acad. Sci. USA* 89:4457-4461).

In an illustrative embodiment, the recombinant phage antibody system (RPAS, Pharmacia Catalog number 27-9400-01) can be easily modified for use in expressing and

screening *pub* combinatorial libraries of the present invention. For instance, the pCANTAB 5 phagemid of the RPAS kit contains the gene which encodes the phage gIII coat protein. The *pub* combinatorial gene library can be cloned into the phagemid adjacent to the gIII signal sequence such that it will be expressed as a gIII fusion protein. After ligation, the 5 phagemid is used to transform competent *E. coli* TG1 cells. Transformed cells are subsequently infected with M13KO7 helper phage to rescue the phagemid and its candidate *pub* gene insert. The resulting recombinant phage contain phagemid DNA encoding a specific candidate *pub* protein, and display one or more copies of the corresponding fusion coat protein. The phage-displayed candidate proteins which are capable of, for example, 10 binding cdc25, are selected or enriched by panning. For instance, the phage library can be panned on glutathione immobilized cdc25-GST fusion proteins, and unbound phage washed away from the cells. The bound phage is then isolated, and if the recombinant phage express at least one copy of the wild type gIII coat protein, they will retain their ability to infect *E. coli*. Thus, successive rounds of reinfection of *E. coli*, and panning will greatly enrich for 15 *pub* homologs which can then be screened for further biological activities in order to differentiate agonists and antagonists.

Consequently, the invention also provides for reduction of the subject *pub* proteins to generate mimetics, e.g. peptide or non-peptide agents, which are able to mimic binding of the authentic protein to another cellular partner. Such mutagenic techniques as described 20 above, as well as the thioredoxin system, are also particularly useful for mapping the determinants of a *pub* protein which participate in protein-protein interactions involved in, for example, binding of the subject proteins to each other. To illustrate, the critical residues 25 of a *pub* protein which are involved in molecular recognition of cdc25 can be determined and used to generate *pub*-derived peptidomimetics which bind to cdc25, and by inhibiting *pub* binding, act to prevent activation of the kinase. By employing, for example, scanning mutagenesis to map the amino acid residues of *pub* which are involved in binding cdc25, peptidomimetic compounds can be generated which mimic those residues in binding to the kinase. For instance, non-hydrolyzable peptide analogs of such residues can be generated using benzodiazepine (e.g., see Freidinger et al., in *Peptides: Chemistry and Biology*, G.R. 30 Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988), azepine (e.g., see Huffman et al., in *Peptides: Chemistry and Biology*, G.R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988), substituted gamma lactam rings (Garvey et al., in *Peptides: Chemistry and Biology*, G.R. Marshall ed., ESCOM Publisher: Leiden, Netherlands, 1988), keto-methylene 35 pseudopeptides (Ewenson et al., (1986) *J. Med. Chem.* 29:295; and Ewenson et al., in *Peptides: Structure and Function* (Proceedings of the 9th American Peptide Symposium) Pierce Chemical Co. Rockland, IL, 1985), β -turn dipeptide cores (Nagai et al., (1985) *Tetrahedron Lett* 26:647; and Sato et al., (1986) *J Chem Soc Perkin Trans* 1:1231), and β -aminoalcohols (Gordon et al., (1985) *Biochem Biophys Res Commun* 126:419; and Dann et al., (1986) *Biochem Biophys Res Commun* 134:71).

Another aspect of the invention pertains to an antibody specifically reactive with a *pub* protein. For example, by using peptides based on the sequence of the subject human or yeast *pub* protein, anti-*pub1* or anti-*pub2* antisera or anti-*pub1* or anti-*pub2* monoclonal antibodies can be made using standard methods. A mammal such as a mouse, a hamster or 5 rabbit can be immunized with an immunogenic form of the peptide (e.g., an antigenic fragment which is capable of eliciting an antibody response). Techniques for conferring immunogenicity on a protein or peptide include conjugation to carriers or other techniques well known in the art. For instance, a peptidyl portion of the protein represented by SEQ ID No. 2, SEQ ID No. 4, SEQ ID No: 6 and/or SEQ ID No. 8 can be administered in the 10 presence of adjuvant. The progress of immunization can be monitored by detection of antibody titers in plasma or serum. Standard ELISA or other immunoassays can be used with the immunogen as antigen to assess the levels of antibodies.

Following immunization, anti-*pub* antisera can be obtained and, if desired, polyclonal anti-*pub* antibodies isolated from the serum. To produce monoclonal antibodies, 15 antibody producing cells (lymphocytes) can be harvested from an immunized animal and fused by standard somatic cell fusion procedures with immortalizing cells such as myeloma cells to yield hybridoma cells. Such techniques are well known in the art, and include, for example, the hybridoma technique (originally developed by Kohler and Milstein, (1975) *Nature*, 256: 495-497), as the human B cell hybridoma technique (Kozbar et al., (1983) 20 *Immunology Today*, 4: 72), and the EBV-hybridoma technique to produce human monoclonal antibodies (Cole et al., (1985) *Monoclonal Antibodies and Cancer Therapy*, Alan R. Liss. Inc. pp. 77-96). Hybridoma cells can be screened immunochemically for production of antibodies specifically reactive with the *pub* proteins and the monoclonal antibodies isolated.

25 The term antibody as used herein is intended to include fragments thereof which are also specifically reactive with an eukaryotic, e.g., mammalian *pub* protein. Antibodies can be fragmented using conventional techniques and the fragments screened for utility in the same manner as described above for whole antibodies. For example, $F(ab')_2$ fragments can be generated by treating antibody with pepsin. The resulting $F(ab')_2$ fragment can be treated to 30 reduce disulfide bridges to produce Fab' fragments. The antibody of the present invention is further intended to include bispecific and chimeric molecules, as well as single chain (scFv) antibodies.

Both monoclonal and polyclonal antibodies (Ab) directed against the subject *pub* protein, and antibody fragments such as Fab' and $F(ab')_2$, can be used to selectively block 35 the action of individual *pub* proteins and allow the study of the cell-cycle or cell proliferation.

Another application of anti-*pub* antibodies is in the immunological screening of cDNA libraries constructed in expression vectors, such as λ gt11, λ gt18-23, λ ZAP, and λ ORF8. Messenger libraries of this type, having coding sequences inserted in the correct

reading frame and orientation, can produce fusion proteins. For instance, λ gt11 will produce fusion proteins whose amino termini consist of β -galactosidase amino acid sequences and whose carboxy termini consist of a foreign polypeptide. Antigenic epitopes of a *pub* protein, such as proteins antigenically related to the h-pub1 protein of SEQ ID No. 2 or s-pub1 of 5 SEQ ID No. 4 or the h-pub2 protein of SEQ ID No. 6 or the h-pub3 protein of SEQ ID No. 8, can then be detected with antibodies, as, for example, reacting nitrocellulose filters lifted from infected plates with an anti-*pub* antibody. Phage, scored by this assay, can then be isolated from the infected plate. Thus, *pub* homologs can be detected and cloned from other sources.

10 Antibodies which are specifically immunoreactive with a *pub* protein of the present invention can also be used in immunohistochemical staining of tissue samples in order to evaluate the abundance and pattern of expression of the protein. Anti-*pub* antibodies can be used diagnostically in immuno-precipitation and immuno-blotting to detect and evaluate levels of one or more *pub* proteins in tissue or cells isolated from a bodily fluid as part of a 15 clinical testing procedure. Diagnostic assays using anti-*pub* antibodies, can include, for example, immunoassays designed to aid in early diagnosis of a neoplastic or hyperplastic disorder, e.g. the presence of cancerous cells in the sample, e.g. to detect cells in which alterations in expression levels of *pub* gene has occurred relative to normal cells.

In addition, nucleotide probes can be generated from the cloned sequence of the 20 subject *pub* proteins which allow for histological screening of intact tissue and tissue samples for the presence of a *pub* protein encoding nucleic acids. Similar to the diagnostic uses of anti-*pub* protein antibodies, the use of probes directed to *pub* protein encoding mRNAs, or to genomic *pub* gene sequences, can be used for both predictive and therapeutic evaluation of allelic mutations which might be manifest in, for example, neoplastic or 25 hyperplastic disorders (e.g. unwanted cell growth) or unwanted differentiation events.

Used in conjunction with anti-*pub* protein antibody immunoassays, the nucleotide probes can help facilitate the determination of the molecular basis for a developmental disorder which may involve some abnormality associated with expression (or lack thereof) of a *pub* protein. For instance, variation in *pub* protein synthesis can be differentiated from a 30 mutation in the coding sequence.

Accordingly, the present method provides a method for determining if a subject is at risk for a disorder characterized by aberrant cell proliferation and/or differentiation. In preferred embodiments, method can be generally characterized as comprising detecting, in a sample of cells from the subject, the presence or absence of a genetic lesion characterized by 35 at least one of (i) an alteration affecting the integrity of a gene encoding a *pub* protein, such as h-pub1, h-pub2 or h-pub3; or (ii) the mis-expression of the *pub* gene. To illustrate, such genetic lesions can be detected by ascertaining the existence of at least one of (i) a deletion of one or more nucleotides from a *pub* gene, (ii) an addition of one or more nucleotides to a *pub* gene, (iii) a substitution of one or more nucleotides of a *pub* gene, (iv) a gross chromosomal

rearrangement of a *pub* gene, (v) a gross alteration in the level of a messenger RNA transcript of a *pub* gene, (vii) aberrant modification of a *pub* gene, such as of the methylation pattern of the genomic DNA, (vii) the presence of a non-wild type splicing pattern of a messenger RNA transcript of a *pub* gene, (viii) a non-wild type level of a *pub* protein, and (ix) inappropriate 5 post-translational modification of a *pub* protein. As set out below, the present invention provides a large number of assay techniques for detecting lesions in a *pub* gene, and importantly, provides the ability to discern between different molecular causes underlying *pub* dependent aberrant cell growth, proliferation and/or differentiation.

Diagnostic assays are also similarly available for detecting s-*pub1* genes, or 10 homologs from other fungus, in order to detect mycotic infections.

In an exemplary embodiment, there is provided a nucleic acid composition comprising a (purified) oligonucleotide probe including a region of nucleotide sequence which is capable of hybridizing to a sense or antisense sequence of a *pub* gene, such as represented by any of SEQ ID Nos: 1, 3, 5 and/or 7, or naturally occurring mutants thereof, 15 or 5' or 3' flanking sequences or intronic sequences naturally associated with the subject *pub* genes or naturally occurring mutants thereof. The nucleic acid of a cell is rendered accessible for hybridization, the probe is exposed to nucleic acid of the sample, and the hybridization of the probe to the sample nucleic acid is detected. Such techniques can be used to detect lesions at either the genomic or mRNA level, including deletions, 20 substitutions,etc., as well as to determine mRNA transcript levels.

In certain embodiments, detection of the lesion comprises utilizing the probe/primer in a polymerase chain reaction (PCR) (see, e.g. U.S. Patent Nos. 4,683,195 and 4,683,202), such as anchor PCR or RACE PCR, or, alternatively, in a ligation chain reaction (LCR) (see, e.g., Landegran et al., (1988) *Science* 241:1077-1080; and Nakazawa et al., (1944) *Proc. Natl. Acad. Sci. USA* 91:360-364), the later of which can be particularly useful for detecting 25 point mutations in the *pub* gene. In a merely illustrative embodiment, the method includes the steps of (i) collecting a sample of cells from a patient, (ii) isolating nucleic acid (e.g., genomic, mRNA or both) from the cells of the sample, (iii) contacting the nucleic acid sample with one or more primers which specifically hybridize to a *pub* gene under conditions 30 such that hybridization and amplification of the *pub* gene (if present) occurs, and (iv) detecting the presence or absence of an amplification product, or detecting the size of the amplification product and comparing the length to a control sample.

In still another embodiment, the level of a *pub* protein can be detected by immunoassay. For instance, the cells of a biopsy sample can be lysed, and the level of a *pub* 35 protein present in the cell can be quantitated by standard immunoassay techniques. In yet another exemplary embodiment, aberrant methylation patterns of a *pub* gene can be detected by digesting genomic DNA from a patient sample with one or more restriction endonucleases that are sensitive to methylation and for which recognition sites exist in the *pub* gene (including in the flanking and intronic sequences). See, for example, Buiting et al.,

(1994) *Human Mol Genet* 3:893-895. Digested DNA is separated by gel electrophoresis, and hybridized with probes derived from, for example, genomic or cDNA sequences. The methylation status of the *pub* gene can be determined by comparison of the restriction pattern generated from the sample DNA with that for a standard of known methylation.

5 Furthermore, the subject gene constructs described above can be utilized in diagnostic assays to determine if a cell's growth is no longer dependent on the regulatory function of a *pub* protein, e.g. in determining the phenotype of a transformed cell. To illustrate, a sample of cells from the tissue can be obtained from a patient and dispersed in appropriate cell culture media. a portion of the cells in the sample can be caused to express a 10 recombinant *pub* protein, e.g. by transfection with an h-pub1, h-pub2, h-pub3 or s-pub1 expression vector, and subsequent growth of the cells assessed. The ability of cells to proliferate despite expression of the *pub* protein is indicative of a lack of dependence on cell regulatory pathways which include the *pub* protein. Depending on the nature of the tissue of 15 interest, the sample can be in the form of cells isolated from, for example, a blood sample, an exfoliated cell sample, a fine needle aspirant sample, or a biopsied tissue sample. Where the initial sample is a solid mass, the tissue sample can be minced or otherwise dispersed so that cells can be cultured, as is known in the art. Such knowledge can have both prognostic and therapeutic benefits.

20 In yet another embodiment, a diagnostic assay is provided which detects the ability of a *pub* gene product, e.g., isolated from a biopsied cell, to bind to other cellular proteins. For instance, it will be desirable to detect h-pub1 mutants which bind with higher binding affinity a cdc25 phosphatase. Such mutants may arise, for example, from fine mutations, e.g., point mutants, which may be impractical to detect by the diagnostic DNA sequencing 25 techniques or by the immunoassays described above. The present invention accordingly further contemplates diagnostic screening assays which generally comprise cloning one or more *pub* genes from the sample cells, and expressing the cloned genes under conditions which permit detection of an interaction between that recombinant gene product and a target protein, e.g., a cdc25 or p53.

30 As will be apparent from the description of the various drug screening assays set forth below, a wide variety of techniques can be used to determine the ability of a *pub* protein to bind to other cellular components, e.g., a cdc25 phosphatase such as cdc25A, cdc25B or cdc25C. These techniques can be used to detect mutations in a *pub* gene which give rise to mutant proteins with a higher or lower binding affinity for a cdc25 relative to the wild-type *pub* gene product. Conversely, by switching which of the cdc25 and *pub* protein is 35 the "bait" and which is derived from the patient sample, the subject assay can also be used to detect cdc25 mutants which have a higher or lower binding affinity for a *pub* protein relative to a wild-type form of that cdc25.

In an exemplary embodiment, cdc25 (e.g. wild-type) can be provided as an immobilized protein (a "bait" or "target"), such as by use of GST fusion proteins and

glutathione-treated microtitre plates. A *pub* gene (a "sample" gene) is amplified from cells of a patient sample, e.g., by PCR, cloned into an expression vector, and transformed into an appropriate host cell. The recombinantly produced *pub* protein is then contacted with the immobilized *cdc25*, e.g., as a lysate or a semi-purified preparation (see *infra*), the complex washed, and the amount of *cdc25/pub* complex determined and compared to a level of wild-type complex formed in a control. Detection can be by, for instance, an immunoassay using antibodies against the wild-type form of the *pub* protein, or by virtue of a label provided by cloning the sample *pub* gene into a vector which provides the protein as a fusion protein including a detectable tag. For example, a *myc* epitope can be provided as part of a fusion protein with the sample *pub* gene. Such fusion proteins can, in addition to providing a detectable label, also permit purification of the sample *pub* protein from the lysate prior to application to the immobilized.

In yet another embodiment of the subject screening assay, the two hybrid assay can be used to detect mutations in either a *pub* gene or *cdc25* gene which alter complex formation between those two proteins (see, for example, U.S. Patent No: 5,283,317; PCT publication WO94/10300; Zervos et al., (1993) *Cell* 72:223-232; Madura et al., (1993) *J Biol Chem* 268:12046-12054; Bartel et al., (1993) *Biotechniques* 14:920-924; and Iwabuchi et al., (1993) *Oncogene* 8:1693-1696). Accordingly, the present invention provides a convenient method for detecting mutants of *pub* genes encoding proteins which are unable to physically interact with a *cdc25* "bait" protein, which method relies on detecting the reconstitution of a transcriptional activator in a *pub/cdc25*-dependent fashion.

Similar embodiments can be derived with other cellular binding partners of the *pub* proteins, such as p53.

Still another aspect of the invention features transgenic non-human animals which express a heterologous *pub* gene of the present invention, or which have had one or more genomic *pub* gene(s) disrupted in at least one of the tissue or cell-types of the animal. For instance, transgenic mice that are disrupted at their *pub* gene locus can be generated.

In another aspect, the invention features an animal model for developmental diseases, which has a *pub* allele which is mis-expressed. For example, a mouse can be bred which has a *pub* allele deleted, or in which all or part of one or more *pub* exons are deleted. Such a mouse model can then be used to study disorders arising from mis-expression of the *pub* gene.

Accordingly, the present invention concerns transgenic animals which are comprised of cells (of that animal) which contain a transgene of the present invention and which preferably (though optionally) express an exogenous *pub* protein in one or more cells in the animal. The *pub* transgene can encode the wild-type form of the protein, or can encode homologs thereof, including both agonists and antagonists, as well as antisense constructs. In preferred embodiments, the expression of the transgene is restricted to specific subsets of cells, tissues or developmental stages utilizing, for example, *cis*-acting sequences that

control expression in the desired pattern. In the present invention, such mosaic expression of the subject protein can be essential for many forms of lineage analysis and can additionally provide a means to assess the effects of, for example, modulation of cdc25 protein levels, and thus activation of a CDK, e.g., cdc2 which might grossly alter development in small patches of tissue within an otherwise normal embryo. Toward this end, tissue-specific regulatory sequences and conditional regulatory sequences can be used to control expression of the transgene in certain spatial patterns. Moreover, temporal patterns of expression can be provided by, for example, conditional recombination systems or prokaryotic transcriptional regulatory sequences.

10 Genetic techniques which allow for the expression of transgenes can be regulated via site-specific genetic manipulation *in vivo* are known to those skilled in the art. For instance, genetic systems are available which allow for the regulated expression of a recombinase that catalyzes the genetic recombination a target sequence. As used herein, the phrase "target sequence" refers to a nucleotide sequence that is genetically recombined by a recombinase.

15 The target sequence is flanked by recombinase recognition sequences and is generally either excised or inverted in cells expressing recombinase activity. Recombinase catalyzed recombination events can be designed such that recombination of the target sequence results in either the activation or repression of expression of the subject *pub* polypeptides. For example, excision of a target sequence which interferes with the expression of a recombinant

20 *pub* gene can be designed to activate expression of that gene. This interference with expression of the protein can result from a variety of mechanisms, such as spatial separation of the *pub* gene from the promoter element or an internal stop codon. Moreover, the transgene can be made wherein the coding sequence of the gene is flanked recombinase recognition sequences and is initially transfected into cells in a 3' to 5' orientation with

25 respect to the promoter element. In such an instance, inversion of the target sequence will reorient the subject gene by placing the 5' end of the coding sequence in an orientation with respect to the promoter element which allow for promoter driven transcriptional activation.

30 In an illustrative embodiment, either the *cre/loxP* recombinase system of bacteriophage P1 (Lakso et al., (1992) *Proc. Natl. Acad. Sci. USA* 89:6232-6236; Orban et al., (1992) *Proc. Natl. Acad. Sci. USA* 89:6861-6865) or the FLP recombinase system of *Saccharomyces cerevisiae* (O'Gorman et al., (1991) *Science* 251:1351-1355; PCT publication WO 92/15694) can be used to generate *in vivo* site-specific genetic recombination systems. Cre recombinase catalyzes the site-specific recombination of an intervening target sequence located between *loxP* sequences. *loxP* sequences are 34 base pair nucleotide repeat sequences to which the Cre recombinase binds and are required for Cre recombinase mediated genetic recombination. The orientation of *loxP* sequences determines whether the intervening target sequence is excised or inverted when Cre recombinase is present (Abremski et al., (1984) *J. Biol. Chem.* 259:1509-1514); catalyzing the excision of the target sequence when the *loxP* sequences are oriented as direct repeats

and catalyzes inversion of the target sequence when *loxP* sequences are oriented as inverted repeats.

Accordingly, genetic recombination of the target sequence is dependent on expression of the Cre recombinase. Expression of the recombinase can be regulated by promoter elements which are subject to regulatory control, e.g., tissue-specific, developmental stage-specific, inducible or repressible by externally added agents. This regulated control will result in genetic recombination of the target sequence only in cells where recombinase expression is mediated by the promoter element. Thus, the activation expression of the *pub* gene can be regulated via regulation of recombinase expression.

Use of the *cre/loxP* recombinase system to regulate expression of a recombinant *pub* protein requires the construction of a transgenic animal containing transgenes encoding both the Cre recombinase and the subject protein. Animals containing both the Cre recombinase and the recombinant *pub* genes can be provided through the construction of "double" transgenic animals. A convenient method for providing such animals is to mate two transgenic animals each containing a transgene, e.g., the *pub* gene and recombinase gene.

One advantage derived from initially constructing transgenic animals containing a *pub* transgene in a recombinase-mediated expressible format derives from the likelihood that the subject protein may be deleterious upon expression in the transgenic animal. In such an instance, a founder population, in which the subject transgene is silent in all tissues, can be propagated and maintained. Individuals of this founder population can be crossed with animals expressing the recombinase in, for example, one or more tissues. Thus, the creation of a founder population in which, for example, an antagonistic *pub* transgene is silent will allow the study of progeny from that founder in which disruption of cell-cycle regulation in a particular tissue or at developmental stages would result in, for example, a lethal phenotype.

Similar conditional transgenes can be provided using prokaryotic promoter sequences which require prokaryotic proteins to be simultaneous expressed in order to facilitate expression of the transgene. Exemplary promoters and the corresponding trans-activating prokaryotic proteins are given in U.S. Patent No. 4,833,080. Moreover, expression of the conditional transgenes can be induced by gene therapy-like methods wherein a gene encoding the trans-activating protein, e.g. a recombinase or a prokaryotic protein, is delivered to the tissue and caused to be expressed, such as in a cell-type specific manner. By this method, the *pub* transgene could remain silent into adulthood until "turned on" by the introduction of the trans-activator.

In an exemplary embodiment, the "transgenic non-human animals" of the invention are produced by introducing transgenes into the germline of the non-human animal. Embryonal target cells at various developmental stages can be used to introduce transgenes. Different methods are used depending on the stage of development of the embryonal target cell. The zygote is the best target for micro-injection. In the mouse, the male pronucleus reaches the size of approximately 20 micrometers in diameter which allows reproducible

injection of 1-2pl of DNA solution. The use of zygotes as a target for gene transfer has a major advantage in that in most cases the injected DNA will be incorporated into the host gene before the first cleavage (Brinster et al., (1985) *Proc. Natl. Acad. Sci. USA* 82:4438-4442). As a consequence, all cells of the transgenic non-human animal will carry the 5 incorporated transgene. This will in general also be reflected in the efficient transmission of the transgene to offspring of the founder since 50% of the germ cells will harbor the transgene. Microinjection of zygotes is the preferred method for incorporating transgenes in practicing the invention.

Retroviral infection can also be used to introduce transgene into a non-human animal. 10 The developing non-human embryo can be cultured *in vitro* to the blastocyst stage. During this time, the blastomeres can be targets for retroviral infection (Jaenisch, R. (1976) *Proc. Natl. Acad. Sci. USA* 73:1260-1264). Efficient infection of the blastomeres is obtained by enzymatic treatment to remove the zona pellucida (*Manipulating the Mouse Embryo*, Hogan eds. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, 1986). The viral vector 15 system used to introduce the transgene is typically a replication-defective retrovirus carrying the transgene (Jahner et al., (1985) *Proc. Natl. Acad. Sci. USA* 82:6927-6931; Van der Putten et al., (1985) *Proc. Natl. Acad. Sci. USA* 82:6148-6152). Transfection is easily and efficiently obtained by culturing the blastomeres on a monolayer of virus-producing cells (Van der Putten, *supra*; Stewart et al., (1987) *EMBO J.* 6:383-388). Alternatively, infection 20 can be performed at a later stage. Virus or virus-producing cells can be injected into the blastocoele (Jahner et al., (1982) *Nature* 298:623-628). Most of the founders will be mosaic for the transgene since incorporation occurs only in a subset of the cells which formed the transgenic non-human animal. Further, the founder may contain various retroviral insertions 25 of the transgene at different positions in the genome which generally will segregate in the offspring. In addition, it is also possible to introduce transgenes into the germ line by intrauterine retroviral infection of the midgestation embryo (Jahner et al., (1982) *supra*).

A third type of target cell for transgene introduction is the embryonal stem cell (ES). ES cells are obtained from pre-implantation embryos cultured *in vitro* and fused with embryos (Evans et al., (1981) *Nature* 292:154-156; Bradley et al., (1984) *Nature* 309:255-30 258; Gossler et al., (1986) *Proc. Natl. Acad. Sci. USA* 83: 9065-9069; and Robertson et al., (1986) *Nature* 322:445-448). Transgenes can be efficiently introduced into the ES cells by DNA transfection or by retrovirus-mediated transduction. Such transformed ES cells can thereafter be combined with blastocysts from a non-human animal. The ES cells thereafter 35 colonize the embryo and contribute to the germ line of the resulting chimeric animal. For review see Jaenisch, R. (1988) *Science* 240:1468-1474.

Methods of making knock-out or disruption transgenic animals are also generally known. See, for example, *Manipulating the Mouse Embryo*, (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y., 1986). Recombinase dependent knockouts can also be generated, e.g. by homologous recombination to insert target sequences, such that

tissue specific and/or temporal control of inactivation of a *pub* gene can be controlled as above.

Yet another aspect of the invention pertains to methods of treating proliferative and/or differentiative disorders which arise from cells which, despite aberrant growth control, still require a *pub*-dependent cdc25 activation for cell growth or a *pub*-dependent p53 half-life. There are a wide variety of pathological cell proliferative conditions for which the *pub* gene constructs, *pub* mimetics and *pub* antagonists, of the present invention can provide therapeutic benefits, with the general strategy being the inhibition of anomalous cell proliferation. For instance, the gene constructs of the present invention can be used as a part 5 of a gene therapy protocol, such as to reconstitute the function of an h-pub1 or h-pub2 proteins, e.g. in a cell in which the protein is misexpressed or in which signal transduction pathways upstream of a *pub* protein are dysfunctional, or to inhibit the function of the wild-type protein, e.g. by delivery of a dominant negative mutant.

To illustrate, cell types which exhibit pathological or abnormal growth presumably 15 dependent at least in part on a function (or dysfunction) of a *pub* protein include various cancers and leukemias, psoriasis, bone diseases, fibroproliferative disorders such as involving connective tissues, atherosclerosis and other smooth muscle proliferative disorders, as well as chronic inflammation. In addition to proliferative disorders, the treatment of differentiative disorders which result from either de-differentiation of tissue due 20 to aberrant reentry into mitosis, or unwanted differentiation due to a failure of a cdc25 phosphatase to appropriately activate certain CDK complexes.

It will also be apparent that, by transient use of gene therapy constructs of the subject 25 *pub* proteins (e.g. agonist and antagonist forms) or antisense nucleic acids, *in vivo* reformation of tissue can be accomplished, e.g. in the development and maintenance of organs. By controlling the proliferative and differentiative potential for different cells, the subject gene constructs can be used to reform injured tissue, or to improve grafting and morphology of transplanted tissue. For instance, *pub* agonists and antagonists can be employed therapeutically to regulate organs after physical, chemical or pathological insult. For example, gene therapy can be utilized in liver repair subsequent to a partial hepatectomy, 30 or to promote regeneration of lung tissue in the treatment of emphysema.

In one aspect of the invention, expression constructs of the subject *pub* proteins may be administered in any biologically effective carrier, e.g. any formulation or composition capable of effectively transfecting cells *in vivo* with a recombinant *pub* gene. Approaches include insertion of the subject gene in viral vectors including recombinant retroviruses, 35 adenovirus, adeno-associated virus, and herpes simplex virus-1, or recombinant bacterial or eukaryotic plasmids. Viral vectors can be used to transfect cells directly; plasmid DNA can be delivered with the help of, for example, cationic liposomes (lipofectin) or derivatized (e.g. antibody conjugated), polylysine conjugates, gramicidin S, artificial viral envelopes or other such intracellular carriers, as well as direct injection of the gene construct or CaPO₄

precipitation carried out *in vivo*. It will be appreciated that because transduction of appropriate target cells represents the critical first step in gene therapy, choice of the particular gene delivery system will depend on such factors as the phenotype of the intended target and the route of administration, e.g. locally or systemically.

5 A preferred approach for *in vivo* introduction of nucleic acid encoding one of the subject proteins into a cell is by use of a viral vector containing nucleic acid, e.g. a cDNA, encoding the gene product. Infection of cells with a viral vector has the advantage that a large proportion of the targeted cells can receive the nucleic acid. Additionally, molecules encoded within the viral vector, e.g., by a cDNA contained in the viral vector, are expressed 10 efficiently in cells which have taken up viral vector nucleic acid.

Retrovirus vectors and adeno-associated virus vectors are generally understood to be the recombinant gene delivery system of choice for the transfer of exogenous genes *in vivo*, particularly into humans. These vectors provide efficient delivery of genes into cells, and the transferred nucleic acids are stably integrated into the chromosomal DNA of the host. A 15 major prerequisite for the use of retroviruses is to ensure the safety of their use, particularly with regard to the possibility of the spread of wild-type virus in the cell population. The development of specialized cell lines (termed "packaging cells") which produce only replication-defective retroviruses has increased the utility of retroviruses for gene therapy, and defective retroviruses are well characterized for use in gene transfer for gene therapy 20 purposes (for a review see Miller, A.D. (1990) *Blood* 76:271). Thus, recombinant retrovirus can be constructed in which part of the retroviral coding sequence (*gag*, *pol*, *env*) has been replaced by nucleic acid encoding a poly polypeptide, rendering the retrovirus replication defective. The replication defective retrovirus is then packaged into virions which can be used to infect a target cell through the use of a helper virus by standard techniques. 25 Protocols for producing recombinant retroviruses and for infecting cells *in vitro* or *in vivo* with such viruses can be found in *Current Protocols in Molecular Biology*, Ausubel, F.M. et al., (eds.) Greene Publishing Associates, (1989). Sections 9.10-9.14 and other standard laboratory manuals. Examples of suitable retroviruses include pLJ, pZIP, pWE and pEM 30 which are well known to those skilled in the art. Examples of suitable packaging virus lines for preparing both ecotropic and amphotropic retroviral systems include ψ Crip, ψ Cre, ψ 2 and ψ Am. Retroviruses have been used to introduce a variety of genes into many different cell types, including neural cells, epithelial cells, endothelial cells, lymphocytes, myoblasts, hepatocytes, bone marrow cells, *in vitro* and/or *in vivo* (see for example Eglitis et al., (1985) *Science* 230:1395-1398; Danos and Mulligan, (1988) *Proc. Natl. Acad. Sci. USA* 85:6460-35 6464; Wilson et al., (1988) *Proc. Natl. Acad. Sci. USA* 85:3014-3018; Armentano et al., (1990) *Proc. Natl. Acad. Sci. USA* 87:6141-6145; Huber et al., (1991) *Proc. Natl. Acad. Sci. USA* 88:8039-8043; Ferry et al., (1991) *Proc. Natl. Acad. Sci. USA* 88:8377-8381; Chowdhury et al., (1991) *Science* 254:1802-1805; van Beusechem et al., (1992) *Proc. Natl. Acad. Sci. USA* 89:7640-7644; Kay et al., (1992) *Human Gene Therapy* 3:641-647; Dai et

al., (1992) *Proc. Natl. Acad. Sci. USA* 89:10892-10895; Hwu et al., (1993) *J. Immunol.* 150:4104-4115; U.S. Patent No. 4,868,116; U.S. Patent No. 4,980,286; PCT Application WO 89/07136; PCT Application WO 89/02468; PCT Application WO 89/05345; and PCT Application WO 92/07573).

5 In choosing retroviral vectors as a gene delivery system for the subject *pub* genes, it is important to note that a prerequisite for the successful infection of target cells by most retroviruses, and therefore of stable introduction of the recombinant *pub* gene, is that the target cells must be dividing. In general, this requirement will not be a hindrance to use of retroviral vectors to deliver agonistic *pub* gene constructs. In fact, such limitation on 10 infection can be beneficial in circumstances wherein the tissue (e.g. nontransformed cells) surrounding the target cells does not undergo extensive cell division and is therefore refractory to infection with retroviral vectors.

Furthermore, it has been shown that it is possible to limit the infection spectrum of retroviruses and consequently of retroviral-based vectors, by modifying the viral packaging 15 proteins on the surface of the viral particle (see, for example PCT publications WO93/25234, WO94/06920, and WO94/11524). For instance, strategies for the modification of the infection spectrum of retroviral vectors include: coupling antibodies specific for cell surface antigens to the viral *env* protein (Roux et al., (1989) *Proc. Natl. Acad. Sci. USA* 86:9079-9083; Julian et al., (1992) *J. Gen Virol* 73:3251-3255; and Goud et al., (1983) *Virology* 20 163:251-254); or coupling cell surface ligands to the viral *env* proteins (Neda et al., (1991) *J. Biol. Chem.* 266:14143-14146). Coupling can be in the form of the chemical cross-linking with a protein or other variety (e.g. lactose to convert the *env* protein to an asialoglycoprotein), as well as by generating fusion proteins (e.g. single-chain antibody/*env* fusion proteins). This technique, while useful to limit or otherwise direct the infection to 25 certain tissue types, and can also be used to convert an ecotropic vector in to an amphotropic vector.

Moreover, use of retroviral gene delivery can be further enhanced by the use of tissue- or cell-specific transcriptional regulatory sequences which control expression of the *pub* gene of the retroviral vector.

30 Another viral gene delivery system useful in the present invention utilizes adenovirus-derived vectors. The genome of an adenovirus can be manipulated such that it encodes a gene product of interest, but is inactivate in terms of its ability to replicate in a normal lytic viral life cycle (see, for example, Berkner et al., (1988) *BioTechniques* 6:616; Rosenfeld et al., (1991) *Science* 252:431-434; and Rosenfeld et al., (1992) *Cell* 68:143-155). 35 Suitable adenoviral vectors derived from the adenovirus strain Ad type 5 dl324 or other strains of adenovirus (e.g., Ad2, Ad3, Ad7 etc.) are well known to those skilled in the art. Recombinant adenoviruses can be advantageous in certain circumstances in that they are not capable of infecting nondividing cells and can be used to infect a wide variety of cell types, including airway epithelium (Rosenfeld et al., (1992) cited *supra*), endothelial cells

(Lemarchand et al., (1992) *Proc. Natl. Acad. Sci. USA* 89:6482-6486), hepatocytes (Herz and Gerard, (1993) *Proc. Natl. Acad. Sci. USA* 90:2812-2816) and muscle cells (Quantin et al., (1992) *Proc. Natl. Acad. Sci. USA* 89:2581-2584). Furthermore, the virus particle is relatively stable and amenable to purification and concentration, and as above, can be modified so as to affect the spectrum of infectivity. Additionally, introduced adenoviral DNA (and foreign DNA contained therein) is not integrated into the genome of a host cell but remains episomal, thereby avoiding potential problems that can occur as a result of insertional mutagenesis in situations where introduced DNA becomes integrated into the host genome (e.g., retroviral DNA). Moreover, the carrying capacity of the adenoviral genome for foreign DNA is large (up to 8 kilobases) relative to other gene delivery vectors (Berkner et al., *supra*; Haj-Ahmand and Graham (1986) *J. Virol.* 57:267). Most replication-defective adenoviral vectors currently in use and therefore favored by the present invention are deleted for all or parts of the viral E1 and E3 genes but retain as much as 80% of the adenoviral genetic material (see, e.g., Jones et al., (1979) *Cell* 16:683; Berkner et al., *supra*; and Graham et al., in *Methods in Molecular Biology*, E.J. Murray, Ed. (Humana, Clifton, NJ, 1991) vol. 7, pp. 109-127). Expression of the inserted *pub* gene can be under control of, for example, the E1A promoter, the major late promoter (MLP) and associated leader sequences, the E3 promoter, or exogenously added promoter sequences.

Yet another viral vector system useful for delivery of the subject *pub* genes is the adeno-associated virus (AAV). Adeno-associated virus is a naturally occurring defective virus that requires another virus, such as an adenovirus or a herpes virus, as a helper virus for efficient replication and a productive life cycle. (For a review, see Muzyczka et al., *Curr. Topics in Micro. and Immunol.* (1992) 158:97-129). It is also one of the few viruses that may integrate its DNA into non-dividing cells, and exhibits a high frequency of stable integration (see for example Flotte et al., (1992) *Am. J. Respir. Cell. Mol. Biol.* 7:349-356; Samulski et al., (1989) *J. Virol.* 63:3822-3828; and McLaughlin et al., (1989) *J. Virol.* 62:1963-1973). Vectors containing as little as 300 base pairs of AAV can be packaged and can integrate. Space for exogenous DNA is limited to about 4.5 kb. An AAV vector such as that described in Tratschin et al., (1985) *Mol. Cell. Biol.* 5:3251-3260 can be used to introduce DNA into cells. A variety of nucleic acids have been introduced into different cell types using AAV vectors (see for example Hermonat et al., (1984) *Proc. Natl. Acad. Sci. USA* 81:6466-6470; Tratschin et al., (1985) *Mol. Cell. Biol.* 4:2072-2081; Wondisford et al., (1988) *Mol. Endocrinol.* 2:32-39; Tratschin et al., (1984) *J. Virol.* 51:611-619; and Flotte et al., (1993) *J. Biol. Chem.* 268:3781-3790).

Other viral vector systems that may have application in gene therapy have been derived from herpes virus, vaccinia virus, and several RNA viruses. In particular, herpes virus vectors may provide a unique strategy for persistence of the recombinant *pub* gene in cells of the central nervous system and ocular tissue (Pepose et al., (1994) *Invest Ophthalmol Vis Sci* 35:2662-2666)

In addition to viral transfer methods, such as those illustrated above, non-viral methods can also be employed to cause expression of a *pub* protein in the tissue of an animal. Most nonviral methods of gene transfer rely on normal mechanisms used by mammalian cells for the uptake and intracellular transport of macromolecules. In preferred 5 embodiments, non-viral gene delivery systems of the present invention rely on endocytic pathways for the uptake of the subject *pub* gene by the targeted cell. Exemplary gene delivery systems of this type include liposomal derived systems, poly-lysine conjugates, and artificial viral envelopes.

In a representative embodiment, a gene encoding a *pub* polypeptide can be entrapped 10 in liposomes bearing positive charges on their surface (e.g., lipofectins) and (optionally) which are tagged with antibodies against cell surface antigens of the target tissue (Mizuno et al., (1992) *No Shinkei Geka* 20:547-551; PCT publication WO91/06309; Japanese patent application 1047381; and European patent publication EP-A-43075). For example, lipofection of neuroglioma cells can be carried out using liposomes tagged with monoclonal 15 antibodies against glioma-associated antigen (Mizuno et al., (1992) *Neurol. Med. Chir.* 32:873-876).

In yet another illustrative embodiment, the gene delivery system comprises an antibody or cell surface ligand which is cross-linked with a gene binding agent such as poly-lysine (see, for example, PCT publications WO93/04701, WO92/22635, WO92/20316, 20 WO92/19749, and WO92/06180). For example, the subject *pub* gene construct can be used to transfect hepatocytic cells *in vivo* using a soluble polynucleotide carrier comprising an asialoglycoprotein conjugated to a polycation, e.g. poly-lysine (see U.S. Patent 5,166,320). It will also be appreciated that effective delivery of the subject nucleic acid constructs via - 25 mediated endocytosis can be improved using agents which enhance escape of the gene from the endosomal structures. For instance, whole adenovirus or fusogenic peptides of the influenza HA gene product can be used as part of the delivery system to induce efficient disruption of DNA-containing endosomes (Mulligan et al., (1993) *Science* 260:926; Wagner et al., (1992) *Proc. Natl. Acad. Sci. USA* 89:7934; and Christiano et al., (1993) *Proc. Natl. Acad. Sci. USA* 90:2122).

In clinical settings, the gene delivery systems can be introduced into a patient by any 30 of a number of methods, each of which is familiar in the art. For instance, a pharmaceutical preparation of the gene delivery system can be introduced systemically, e.g. by intravenous injection, and specific transduction of the construct in the target cells occurs predominantly from specificity of transfection provided by the gene delivery vehicle, cell-type or tissue-type expression due to the transcriptional regulatory sequences controlling expression of the gene, or a combination thereof. In other embodiments, initial delivery of the recombinant 35 gene is more limited with introduction into the animal being quite localized. For example, the gene delivery vehicle can be introduced by catheter (see U.S. Patent 5,328,470) or by stereotactic injection (e.g. Chen et al., (1994) *Proc. Natl. Acad. Sci. USA* 91: 3054-3057).

Moreover, as set out above, the present invention also provides assays for identifying drugs which are either agonists or antagonists of the normal cellular function of *pub* proteins, or of the role of *pub* proteins in the pathogenesis of normal or abnormal cellular proliferation and/or differentiation and disorders related thereto, as mediated by, for example, binding of *pub* to a target protein, e.g., a mitotic activating tyrosine phosphatase such as cdc25, or a tumor suppressor protein such as p53. In one embodiment, the assay evaluates the ability of a compound to modulate binding and/or ubiquitinylation of a cdc25 or p53 protein or other complexes of cell-cycle regulatory proteins by a *pub* protein of the present invention. While the following description is directed generally to embodiments exploiting the interaction between *pub1* and cdc25, it will be understood that similar embodiments can be generated using, for example, a *pub2* or *pub3* protein and cdc25, or either a *pub1* or *pub2* protein and other cell-cycle regulatory proteins such as p53.

A variety of assay formats will suffice and, in light of the present disclosure, those not expressly described herein will nevertheless be comprehended by one of ordinary skill in the art. Agents to be tested for their ability to act as *pub* inhibitors can be produced, for example, by bacteria, yeast or other organisms (e.g. natural products), produced chemically (e.g. small molecules, including peptidomimetics), or produced recombinantly. In a preferred embodiment, the test agent is a small organic molecule, e.g., other than a peptide, oligonucleotide, or analog thereof, having a molecular weight of less than about 2,000 daltons.

Assays which approximate the ubiquitination of target regulatory proteins in eukaryotic cells, particularly mammalian cells, can be generated in many different forms, and include assays based on cell-free systems, e.g. purified proteins or cell lysates, as well as cell-based assays which utilize intact cells. Assays as described herein can be used in conjunction with the subject E3-like complexes to generate a ubiquitin-conjugating system for detecting agents able to modulate particular *pub*-dependent ubiquitination of cellular or viral regulatory proteins. Such modulators can be used, for example, in the treatment of proliferative and/or differentiative disorders, to modulate apoptosis, and in the treatment of viral infections.

In many drug screening programs which test libraries of compounds and natural extracts, high throughput assays are desirable in order to maximize the number of compounds surveyed in a given period of time. Assays of the present invention which are performed in cell-free systems, such as may be derived with purified or semi-purified proteins or with lysates, are often preferred as "primary" screens in that they can be generated to permit rapid development and relatively easy detection of an alteration in a molecular target which is mediated by a test compound. Moreover, the effects of cellular toxicity and/or bioavailability of the test compound can be generally ignored in the *in vitro* system, the assay instead being focused primarily on the effect of the drug on the molecular target as may be manifest in an alteration of binding affinity with other proteins or change in

enzymatic properties of the molecular target. Accordingly, potential modifiers, e.g., activators or inhibitors of *pub*-dependent ubiquitination of a target protein can be detected in a cell-free assay generated by constitution of a functional ubiquitin conjugating system in a cell lysate, such as generated by charging a ubiquitin-depleted reticulocyte lysate (Hershko et al., (1983) *J Biol Chem* 258:8206-8214) with one or more of a ubiquitin-conjugating enzyme, an E1 enzyme, an E3-like complex comprising *pub1*, ubiquitin, and/or a substrate for *pub1*-dependent ubiquitination, such as a cdc25 phosphatase. In an alternate format, the assay can be derived as a reconstituted protein mixture which, as described below, offers a number of benefits over lysate-based assays.

In an illustrative embodiment of the present assay, the ubiquitin-conjugating system comprises a reconstituted protein mixture of at least semi-purified proteins, and even more preferably of purified proteins. The reconstituted protein mixture is derived from preparations of the regulatory protein and ubiquitin under conditions which drive the conjugation of the two molecules. For instance, the mixture can include a ubiquitin-activating enzyme (E1), a ubiquitin-conjugating enzyme (E2), an E3-like complex comprising *pub1*, and a nucleotide triphosphate (e.g. ATP). Alternatively, the E1 enzyme, the ubiquitin, and the nucleotide triphosphate can be substituted in the system with a pre-activated ubiquitin in the form of an E1:Ub conjugate. Likewise, a pre-activated ubiquitin can instead comprise an E2:Ub conjugate.

In preferred embodiments, the purified protein mixture substantially lacks any proteolytic activity which would degrade the target protein and/or components of the ubiquitin conjugating system. For instance, the reconstituted system can be generated to have less than 10% of the proteolytic activity associated with a typical reticulocyte lysate, and preferably no more than 5%, and most preferably less than 2%. Alternatively, the mixture can be generated to include, either from the onset of ubiquitination or from some point after ubiquitin conjugation of the regulatory protein, a ubiquitin-dependent proteolytic activity, such as a purified proteosome complex, that is present in the mixture at measured amounts.

In general, the use of reconstituted protein mixtures will be preferred among cell-free embodiments of the subject assay because they allow more careful control of the reaction conditions in the ubiquitin-conjugating system. Moreover, the system can be derived to favor discovery of modifiers, e.g., activators or inhibitors of particular steps of the ubiquitination process, especially the *pub1*-dependent steps. For instance, as set out above, a reconstituted protein assay can be generated which does not facilitate degradation of the ubiquitinated protein, and which utilizes a precharged E2:Ub conjugate. The level of ubiquitin-conjugated protein, which is dependent on an E3-like complex can easily be measured directly in such a system, both in the presence and absence of a candidate agent, thereby enhancing the ability to detect a modifier of the *pub1*-dependent step. Alternatively, the Ub-conjugating system can be allowed to develop a steady state level of regulatory

protein:Ub conjugates in the absence of a proteolytic activity, but then shifted to a degradative system by addition of purified Ub-dependent proteases. Such degradative systems would be amenable to identifying proteosome inhibitors.

Moreover, in the subject method, ubiquitin conjugating systems derived from purified 5 proteins hold a number of significant advantages over cell lysate or wheat germ extract based assays (collectively referred to hereinafter as "lysates"), especially "whole" lysates. Unlike the reconstituted protein system, the synthesis and destruction of the target protein cannot be readily controlled for in lysate-based assays. Without knowledge of particular kinetic parameters for Ub-independant and Ub-dependent degradation of the target protein in the 10 lysate, discerning between the two pathways can be extremely difficult. Measuring these parameters, if at all possible, is further made tedious by the fact that cell lysates tend to be inconsistent from batch to batch, with potentially significant variation between preparations. Evaluation of a potential inhibitor using a lysate system is also complicated in those 15 circumstances where the lysate is charged with mRNA encoding the target protein, as such lysates may continue to synthesize the protein during the assay, and will do so at unpredictable rates.

Accordingly, knowledge of the concentration of each component of the ubiquitin 20 conjugation pathway can be required for each lysate batch, along with the degradative kinetic data, in order to determine the necessary time course and calculate the sensitivity of experiments performed from one lysate preparation to the next.

Furthermore, the lysate system can be unsatisfactory where the target protein itself has a 25 relatively short half-life, especially if due to degradative processes other than the ubiquitin-mediated pathway to which an inhibitor is sought. However, as described, this effect can be mitigated by the use of protease inhibitors such as PMSF or TPCK to inhibit proteolysis of the target protein, though broad-spectrum inhibitors will knock out both ubiquitin-dependent and independent proteolysis.

Moreover, many of the disadvantages of whole cell lysates described above can be overcome by the use of semi-purified cell extracts and/or lysates that have been charged with 30 one or more components of a ubiquitin-conjugation pathway. For example, by selective removal of cell lysate components which interfere with ubiquitination assays, an assay may be feasible in a cell extract even without further purification. Such an approach makes possible rapid and inexpensive development of assay systems suitable for use with ubiquitination assays.

Thus, in another aspect of the subject invention, the ubiquitin-conjugating system 35 comprises a semi-purified cell extract. For instance, as described in the examples below, semi-purified cell extracts can be produced by treatment of cell lysates by a variety of techniques. For example, chromatographic methods and the like can be used to partially purify at least one component of the cell lysate. Likewise, semi-purified cell lysates may be prepared by treatment of a cell lysate to selectively remove a component of the lysate, for

example, by immunoprecipitation. Many other methods for the preparation of semi-purified cell extracts by the selective removal or enrichment of components of a cell lysate will be evident to the skilled artisan.

In yet another embodiment of the subject assay, a cell lysate can be charged with 5 certain of the components of a *pub1*-dependent ubiquitination system. For example, in addition to inhibitors or potentiators of ubiquitination, a semi-purified cell extract can be charged with the relevant UBC, *pub1*, cdc25 phosphatase and the like. Likewise, lysates can be generated from cells recombinantly manipulated to produce, for example, a labeled component to the assay, such as a myc-labeled ubiquitin or a GST-cdc25 fusion protein.

10 Ubiquitination of the target regulatory protein via an *in vitro* ubiquitin-conjugating system, in the presence and absence of a candidate inhibitor, can be accomplished in any vessel suitable for containing the reactants. Examples include microtitre plates, test tubes, and micro-centrifuge tubes. In certain embodiments of the present assay, the *in vitro* assay system is generated to lack the ability to degrade the ubiquitinated target protein. In such an 15 embodiments, a wide range of detection means can be practiced to score for the presence of the ubiquitinated protein.

20 In one embodiment of the present assay, the products of a non-degradative ubiquitin-conjugating system are separated by gel electrophoresis, and the level of ubiquitinated target protein assessed, using standard electrophoresis protocols, e.g., by detecting an increase in molecular weight of the target protein that corresponds to the addition of one or more ubiquitin chains. For example, one or both of the target protein and ubiquitin can be labeled 25 with a radioisotope such as ³⁵S, ¹⁴C, or ³H, and the isotopically labeled protein bands quantified by autoradiographic techniques. Standardization of the assay samples can be accomplished, for instance, by adding known quantities of labeled proteins which are not themselves subject to ubiquitination or degradation under the conditions which the assay is performed. Similarly, other means of detecting electrophoretically separated proteins can be employed to quantify the level of ubiquitination of the regulatory protein, including 30 immunoblot analysis using antibodies specific for either the regulatory protein or ubiquitin, or derivatives thereof. As described below, the antibody can be replaced with another molecule able to bind one of either the regulatory protein or ubiquitin. By way of illustration, one embodiment of the present assay comprises the use of biotinylated ubiquitin in the conjugating system. The biotin label is detected in a gel during a subsequent detection step by contacting the electrophoretic products (or a blot thereof) with a streptavidin-conjugated label, such as a streptavidin linked fluorochrome or enzyme, which can be readily 35 detected by conventional techniques. Moreover, where a reconstituted protein mixture is used (rather than a lysate) as the conjugating system, it may be possible to simply detect the regulatory protein and ubiquitin conjugates in the gel by standard staining protocols, including coomassie blue and silver staining.

In another embodiment, an immunoassay or similar binding assay, is used to detect and quantify the level of ubiquitinated regulatory protein produced in the ubiquitin-conjugating system. Many different immunoassay techniques are amenable for such use and can be employed to detect and quantitate the regulatory protein:Ub conjugates. For example, the wells of a microtitre plate (or other suitable solid phase) can be coated with an antibody which specifically binds one of either the regulatory protein or ubiquitin. After incubation of the ubiquitin-conjugated system with and without the candidate agent, the products are contacted with the matrix bound antibody, unbound material removed by washing, and ubiquitin conjugates of the regulatory protein specifically detected. To illustrate, if an antibody which binds the regulatory protein is used to sequester the protein on the matrix, then a detectable anti-ubiquitin antibody can be used to score for the presence of ubiquitinated regulatory protein on the matrix.

However, it will be clear to those skilled in the art that the use of antibodies in these binding assays is merely illustrative of binding molecules in general, and that the antibodies are readily substituted in the assay with any suitable molecule that can specifically detect one of either the regulatory protein or the ubiquitin. As described below, a biotin-derivative of ubiquitin can be used, and streptavidin (or avidin) employed to bind the biotinylated ubiquitin. In an illustrative embodiment, wells of a microtitre plate are coated with streptavidin and contacted with the developed ubiquitin-conjugating system under conditions wherein the biotinylated ubiquitin binds to and is sequestered in the wells. Unbound material is washed from the wells, and the level of regulatory protein (bound to the matrix via a conjugated ubiquitin moiety) is detected in each well. Alternatively, the microtitre plate wells can be coated with an antibody (or other binding molecule) which binds and sequesters the regulatory protein on the solid support, and detection of ubiquitinated conjugates of the matrix-bound regulatory protein are subsequently carried out using a detectable streptavidin derivative, such as an alkaline phosphatase/streptavidin complex.

In similar fashion, epitope-tagged ubiquitin, such as myc-ub (see Ellison et al. (1991) *J. Biol. Chem.* 266:21150-21157; ubiquitin which includes a 10-residue sequence encoding a protein of c-myc) can be used in conjunction with antibodies to the epitope tag. A major advantage of using such an epitope-tagged ubiquitin approach for detecting Ub:protein conjugates is the ability of an N-terminal tag sequences to inhibit ubiquitin-mediated proteolysis of the conjugated regulatory protein.

Other ubiquitin derivatives include detectable labels which do not interfere greatly with the conjugation of ubiquitin to the regulatory protein. Such detectable labels can include fluorescently-labeled (e.g. FITC) or enzymatically-labeled ubiquitin fusion proteins. These derivatives can be produced by chemical cross-linking, or, where the label is a protein, by generation of a fusion protein. Several labeled ubiquitin derivatives are commercially available.

Likewise, other binding molecules can be employed in place of the antibodies that bind the regulatory protein. For example, the regulatory protein can be generated as a glutathione-S-transferase (GST) fusion protein. As a practical matter, such GST fusion protein can enable easy purification of the regulatory protein in the preparation of components of the ubiquitin-conjugating system (see, for example, *Current Protocols in Molecular Biology*, eds. Ausubel *et al.* (NY: John Wiley & Sons, 1991); Smith *et al.* (1988) *Gene* 67:31; and Kaelin *et al.* (1992) *Cell* 70:351) Moreover, glutathione derivatized matrices (e.g. glutathione-sepharose or glutathione-coated microtitre plates) can be used to sequester free and ubiquitinated forms of the regulatory protein from the ubiquitin-conjugating system, and the level of ubiquitin immobilized can be measured as described. Likewise, where the matrix is generated to bind ubiquitin, the level of sequestered GST-regulatory protein can be detected using agents which bind to the GST moiety (such as anti-GST antibodies), or, alternatively, using agents which are enzymatically acted upon by GST to produce detectable products (e.g. 1-chloro-2,4-dinitrobenzene: Habig *et al.* (1974) *J Biol Chem* 249:7130). Similarly, other fusion proteins involving the regulatory protein and an enzymatic activity are contemplated by the present method. For example, fusion proteins containing β -galactosidase or luciferase, to name but a few, can be employed as labels to determine the amount of regulatory protein sequestered on a matrix by virtue of a conjugated ubiquitin chain.

Moreover, such enzymatic fusion proteins can be used to detect and quantitate ubiquitinated regulatory protein in a heterogeneous assay, e.g., one which does not require separation of the components of the conjugating system. For example, ubiquitin conjugating systems can be generated to have a ubiquitin-dependent protease which degrades the regulatory protein. The enzymatic activity of undegraded fusion protein provides a detectable signal, in the presence of substrate, for effectively measuring the level of the regulatory protein ubiquitination. Similarly, in a non-degradative conjugating system, ubiquitination of the regulatory protein portion of the fusion protein can allosterically influence the enzymatic activity associated with the fusion protein and thereby provides a means for monitoring the level of ubiquitin conjugation.

In binding assay-type detection steps such as set out above, the choice of which of either the regulatory protein or ubiquitin should be specifically sequestered on the matrix will depend on a number of factors, including the relative abundance of both components in the conjugating system. For instance, where the reaction conditions of the ubiquitin conjugating system provide ubiquitin at a concentration far in excess of the level of the regulatory protein, (e.g., one order of magnitude or greater) sequestering the ubiquitin and detecting the amount of regulatory protein bound with the ubiquitin can provide less dynamic range to the detection step of the present method than the converse embodiment of sequestering the regulatory protein and detecting ubiquitin conjugates from the total regulatory protein bound to the matrix. That is, where ubiquitin is provided in great excess

relative to the regulatory protein, the percentage of ubiquitin conjugated regulatory protein in the total ubiquitin bound to the matrix can be small enough that any diminishment in ubiquitination caused by a modifier can be made difficult to detect by the fact that, for example, the statistical error of the system (e.g. the noise) can be a significant portion of the measured change in concentration of bound regulatory protein. Furthermore, it is clear that manipulating the reaction conditions and reactant concentrations in the ubiquitin-conjugating system can be carried out to provide, at the detection step, greater sensitivity by ensuring that a strong ubiquitinated protein signal exists in the absence of any modifier.

In still further embodiments of the present invention, the ubiquitin-conjugating system is generated in whole cells, taking advantage of cell culture techniques to support the subject assay. For example, as described below, the ubiquitin-conjugating system (including the target protein and detection means) can be constituted in a eukaryotic cell culture system, including mammalian and yeast cells. Advantages to generating the subject assay in an intact cell include the ability to detect inhibitors which are functional in an environment more closely approximating that which therapeutic use of the inhibitor would require, including the ability of the agent to gain entry into the cell. Furthermore, certain of the *in vivo* embodiments of the assay, such as examples given below, are amenable to high through-put analysis of candidate agents.

The components of the ubiquitin-conjugating system, including the regulatory protein, can be endogenous to the cell selected to support the assay. Alternatively, some or all of the components can be derived from exogenous sources. For instance, a recombinantly produced E2 enzyme, such as UBC3, UBC4, UBC5 and/or UBC9, or recombinantly produced components of an E3-like complex comprising *pub1*, can be expressed in the cell by recombinant techniques (such as through the use of an expression vector), as well as by microinjecting the proteins themselves or mRNA encoding the protein.

In any case, the cell is ultimately manipulated after incubation with a candidate inhibitor in order to facilitate detection of ubiquitination or ubiquitin-mediated degradation of the regulatory protein. As described above for assays performed in reconstituted protein mixtures or lysates, the effectiveness of a candidate inhibitor can be assessed by measuring direct characteristics of the regulatory protein, such as shifts in molecular weight by electrophoretic means or detection in a binding assay. For these embodiments, the cell will typically be lysed at the end of incubation with the candidate agent, and the lysate manipulated in a detection step in much the same manner as might be the reconstituted protein mixture or lysate.

Indirect measurement of ubiquitination of the target protein can also be accomplished by detecting a biological activity associated with the regulatory protein that is either attenuated by ubiquitin-conjugation or destroyed along with the regulatory protein by ubiquitin-dependent proteolytic processes. As set out above, the use of fusion proteins comprising the regulatory protein and an enzymatic activity are representative embodiments

of the subject assay in which the detection means relies on indirect measurement of ubiquitination of the regulatory protein by quantitating an associated enzymatic activity.

Where the regulatory protein has a relatively short half-life due to ubiquitin-dependent or independent degradation in the cell, preferred embodiments of the assay either 5 do not require cell lysis, or, alternatively, generate a longer lived detection signal that is independent of the regulatory protein's fate after lysis of the cell. With respect to the latter embodiment, the detection means can comprise, for example, a reporter gene construct which includes a positive transcriptional regulatory element that binds and is responsive to the regulatory protein. For instance, where the regulatory protein does not itself possess 10 DNA-binding ability, it can be arranged as part of an interaction trap assay designed for detecting modifiers, e.g., activators or inhibitors, of the *pub1*-dependent destruction of the protein (see, for example, U.S. Patent No: 5,283,317; PCT publication WO94/10300; Zervos et al., (1993) *Cell* 72:223-232; Madura et al., (1993) *J Biol Chem* 268:12046-12054; Bartel et al., (1993) *Biotechniques* 14:920-924; and Iwabuchi et al., (1993) *Oncogene* 8:1693- 15 1696). In an illustrative embodiment, *Saccharomyces cerevisiae* YPB2 cells are transformed simultaneously with a plasmid encoding a GAL4db-*pub1* (where *pub* is a catalytically inactive) fusion and with a plasmid encoding the GAL4ad domain fused to human cdc25 phosphatase. Moreover, the strain is transformed such that the GAL4-responsive promoter drives expression of a phenotypic marker. For example, the ability to grow in the absence of 20 histidine depends on the expression of the HIS3 gene if it is under control of a GAL4-responsive promoter and, therefore, indicates that a functional GAL4 activator has been reconstituted through the interaction of the h-*pub1* and the human cdc25 fusion proteins.

Thus, for example, agents able to inhibit the ubiquitination of the cdc25 fusion protein will result in yeast cells able to growth in the absence of histidine, as the GAL4db- 25 *pub1* and GAL4ad-cdc25 fusion proteins will be able to interact and cause expression of the HIS3 gene. Alternatively, the agents which do not effect the ubiquitination of the cdc25 fusion protein will result in cells unable to grow in the absence of histidine as the GAL4ad-cdc25 fusion protein will be degraded or otherwise prevented from interacting with the GAL4db-*pub1* protein.

30 The present invention also makes available *S. pombe* strains which contain a null *pub* mutation. As described herein, these strains can be complemented using human genes, and thus "humanized" yeast strains can be created for *in vivo* drug screen, e.g., which comprise a human *pub* homolog and (optionally) a human cdc25 phosphatase. The strain can be further manipulated to be "humanized" with respect to other biochemical steps in the *pub1*-mediated 35 ubiquitination of the cdc25 fusion protein. For example, conditional inactivation of the relevant yeast UBC enzyme with concomitant expression of the human UBC homolog, or alternatively, replacement of other yeast genes involved in ubiquitination with their human homologs, provides a humanized system whereby the cdc25 protein can be ubiquitinated by

a *pub1*-dependent mechanism which approximates the *pub1*-dependent ubiquitination that occurs in vertebrate cells.

Furthermore, drug screening assays can be generated which do not measure ubiquitination *per se*, but rather detect inhibitory agents on the basis of their ability to interfere with binding of one of the proteins involved in the *pub1*-dependent ubiquitin conjugation pathway. In an exemplary binding assay, the compound of interest is contacted with a mixture generated from an isolated and purified E2 protein and an E3-like complex comprising the *pub* protein. Alternatively, *pub* and *cdc25* are combined in the presence and absence of test agents so as to provide a competitive binding assay which detects agents able to compete with, or potentiate, the *cdc25* binding to *pub1*. Detection and quantification of complexes between the *pub* and *cdc25* provides a means for determining the compound's efficacy at inhibiting (or potentiating) complex formation between the *pub* and other components of the *pub1*-dependent ubiquitin pathway. The efficacy of the compound can be assessed by generating dose response curves from data obtained using various concentrations of the test compound. Moreover, a control assay can also be performed to provide a baseline for comparison. In the control assay, isolated and purified *cdc25* is added to a composition containing the *pub* protein, and the formation of complexes is quantitated in the absence of the test compound.

Complex formation between *cdc25* protein or other regulatory protein and *pub* may be detected by a variety of techniques, many of which are effectively described above. For instance, modulation in the formation of complexes can be quantitated using, for example, detectably labeled proteins (e.g. radiolabelled, fluorescently labelled, or enzymatically labelled), by immunoassay, or by chromatographic detection.

Typically, it will be desirable to immobilize either the regulatory protein, e.g., *cdc25* or a component of the E3-like complex, such as the *pub* protein, to facilitate separation of complexes from uncomplexed forms of one of the proteins, as well as to accommodate automation of the assay. In an illustrative embodiment, a fusion protein can be provided which adds a domain that permits the protein to be bound to an insoluble matrix. For example, GST/*cdc25* fusion proteins can be adsorbed onto glutathione sepharose beads (Sigma Chemical, St. Louis, MO) or glutathione derivatized microtitre plates, which are then combined with the *pub* protein, e.g. containing ^{35}S -labelled proteins, and the test compound and incubated under conditions conducive to complex formation. Following incubation, the beads are washed to remove any unbound *pub1*, and the matrix bead-bound radiolabel determined directly (e.g. beads placed in scintilant), or in the supernatant after the complexes are dissociated, e.g. when microtitre plate is used. Alternatively, after washing away unbound protein, the complexes can be dissociated from the matrix, separated by SDS-PAGE gel, and the level of, for example, *pub* protein found in the matrix-bound fraction quantitated from the gel using standard electrophoretic techniques.

Exemplification

The invention now being generally described, it will be more readily understood by reference to the following examples which are included merely for purposes of illustration of 5 certain aspects and embodiments of the present invention, and are not intended to limit the invention.

I. Cloning of Schizosaccharomyces pombe Publ

10 In a screen that was originally designed to identify novel *S. pombe* tyrosine kinases, a *S. pombe* cDNA expression library cloned in λ ZAP2 was screened with an antiphosphotyrosine monoclonal antibody (Lindberg et al., (1988) *Oncogene* 3:629-633; Lindberg and Pasquale, (1991) *Meth. Enzymol.* 200:557-564; Druker et al., (1989) *New Eng. J. Med.* 321:1383-1391). Two hundred and fifty thousand phage plaques were examined and 15 two positives obtained, each containing a different cDNA. *Publ* was one of the two genes identified by this screen. The original isolate encoded a fusion protein between the first 24 amino acids of beta-galactosidase and the last 739 amino acids of *publ*. Sequence analysis of this open reading frame revealed a putative ATP binding site (GxGxxG) with a valine residue located 6 and a lysine residue located 25 amino acids downstream of this site (see 20 SEQ ID No. 4). Aside from this potential match to subdomains 1 and 2, *publ* contained no other significant homology to the 12 subdomain consensus sequence of the kinase catalytic domain (Hanks et. al., (1988) *Science* 241:42-52; Hanks and Quinn, (1991) *Meth. Enzymol.* 200:38-62). In addition, we were unable to detect any tyrosine kinase activity associated 25 with *publ* *in vitro*. Peptide and polyglutyltyrosine kinase assays of lysates prepared from cells expressing the original, β gal-*publ* fusion protein were negative (Braun et al., (1984) *J. Biol. Chem.* 259:2051-2054; Casnelli, (1991) *Meth. Enzymol.* 200: ; Racker, (1991) *Meth. Enzymol.* 200:107-111. Wong and Goldberg, (1983) *J. Biol. Chem.* 258:1022-1025). Similarly, peptide, polyglutyltyrosine and autophosphorylation assays of a purified GST-*publ* 30 fusion protein which fused the originally isolated *publ* fragment to the C terminus of glutathione-S-transferase were negative. Finally, these same assays performed with a purified full length *publ* fused to the C terminus of maltose binding protein were also negative. We therefore suspect that the appearance of tyrosine phosphorylated proteins upon expression of *publ* in *E. coli* was indirect.

To obtain a full length *publ* cDNA, the *S. pombe* cDNA library was reprobed with 35 the originally isolated *publ* fragment. A 2,847 bp cDNA was isolated. A single 2.9 kb *publ* message was detected by Northern blot of logarithmically growing wild type cells (972) indicating that we had isolated a cDNA at or close to full length.

To physically map the *publ* gene we probed a collection of contiguous cosmid clones spanning the *S. pombe* genome with *publ* cDNA (Mizukami et al., (1993) *Cell* 73:121-132).

Three overlapping cosmids, 323, 437 and 1187 hybridized to our probe indicating that *pub1* is on the right arm of chromosome 1 near the centromere. Two nearby adjacent NotI sites and cut7 are the closest distal markers to the gene.

5 *II. Pub1 is shares some homology to the putative E6-AP catalytic domain*

Sequence analysis of the 2.8 kb *pub1* cDNA revealed a 766 amino acid open reading frame. *In vitro* transcription and translation of this cDNA resulted in the formation of a 85 kD translation product, consistent with the size of the predicted ORF. A blast search of 10 genbank revealed three proteins of unknown function which share homology with *pub1*: the *S. cerevisiae* protein RSP5 (71% identical), the human protein D42055 (47% identical) and the mouse protein NEDD4 (40% identical) (Kumar et. al.. (1992) *Biochem. Biophys. Res. Comm.* 185:1155-1161; Huijbregtse et al., (1995) 92:2563-2567). The human protein ubiquitin ligase E6-AP is 32% identical to *pub1* and the most similar protein of known 15 function in the database. The protein ubiquitin ligase activity of E6-AP requires the formation of an thioester intermediate between cys 833 of E6-AP and the C terminus of ubiquitin (Scheffner et al. (1995) *Nature* 373:81-83). The site of E6AP thiol ubiquitination and the region surrounding this residue are conserved in *pub1*. The sequence similarity between these two proteins is concentrated in the C terminal third of each. *Pub1* lacks the 20 sequence required for E6 binding and has only slight similarity to the region of E6AP shown to be required for pS3 binding by delctioll analysis (Huijbregtse et al. (1993b) *Mol. Cell. Biol.* 13:4918-4927). The nine proteins identified by a blast search of genbank to be most 25 similar to *pub1* have the same conserved C terminal domain in common with E6AP. These proteins come from several eucaryotic sources and may define an E6AP like family of protein ubiquitin ligases (Scheffner et. al.. (1995) *supra*).

There is a class of proteins which alternate between freely soluble and membrane bound forms in a Ca^{+2} dependent fashion. Such proteins translocate to specific phospholipid membranes in the presence of micromolar amounts of calcium. A peptide containing the sequence necessary and sufficient for the Ca^{+2} dependent phospholipid 30 membrane binding was identified in a cytosolic phospholipase A2 (Clark et al., (1991) *Cell* 65:1043-1051). This peptide contained a sequence motif (CaLB domain) conserved in several proteins which translocate to the plasma membrane in a Ca^{+2} dependent fashion including PKC and GAP (Clark et al., (1991) *supra*). *Pub1* contains a motif which is highly 35 homologous to the CaLB consensus sequence. A full length MBP-*pub1* fusion protein dramatically increases its affinity for hydrophobic column matrices in a Ca^{+2} dependent fashion suggesting that this motif is functional *in vitro*.

III. *Genetic interactions with weel and cdc25*

A *pub 1* null allele was constructed by replacing a 948bp Sal I-Nsi I fragment of the *pub 1* ORF with the *ura4* gene. This construct effectively disrupts about two thirds of the *pub 1* ORF including the putative protein ubiquitin ligase domain. A linear fragment containing the disrupted *pub1* gene was introduced into the diploid strain SP826 (Table 1).

5 Stable Ura⁺ transformants were recovered by screening for failure to grow in the presence of 5-fluoroorotic acid (FOA) and Southern blot analysis confirmed that most of those carried one copy of the *pub1* disruption allele (*pub1::ura4*) and one copy of the wild type gene. Upon sporulation at 30°C the diploid heterozygous for the *pub1* disruption produced four viable spores indicating that *pub1* is not essential for vegetative growth. Southern blot

10 analysis of the Ura⁺ haploid cells confirmed that they carried only the disrupted *pub1* gene.

Table 1
List of *S. pombe* Strains

5	Strain Genotype
	972 h-S
SP 6	h-S leu 1-32
SP 546	h ⁺ N wee1-50
SP 628	h ⁺ N cdc25-22 leu1-32
10 SP826	h ⁺ N/h ⁺ N ade6-210/ade6-216 leu1-32/leu1-32 ura4-D18/ura4-D18
SP 974	h ⁺ N cdc2-3w cdc25-22 ura4-D18
SP 1062	h-S cdc25-22ΔART8-25.4 leu1-32 ura4-
SP 1207	h-S pub1::ura4 ura4-D18
SP 1208	h ⁺ N pub1::ura4 leu1-32 ura4-D18
15 SP 1209	h-S pub1::ura4 wee1-50 ura4-D18
SP 1210	h-S pub1::ura4 cdc25-22 ura4-D18
SP 1211	h-S pub1::ura4 wee1-50 cdc25-22 ura4-D18
SP 1212	h-S mts2 cdc25(HA) ₃ leu1-32
SP 1213	h-S mts2 pub1::ura4 cdc25(HA) ₃ leu1-32 ura4-D18
20	Strain SP 1062 was constructed by stably transforming SP 586 h-S cdc25-22, leu1-32, ura4- with pART8-25.4 which carries cdc25 under the strong constitutive ADH promoter control. StrainCFX 109-2Rh+Ncdc25(HA)3 leu1-32,ura4-D18 used to construct strains SP 1212 and SP 1213. The cdc25 allele cdc25(HA) ₃ has a triple tandem hemagglutinin epitope inserted in frame at the C ₁ site of cdc25. The resulting construct was integrated into the <i>S. pombe</i> genome at the cdc25-22 site. This allele rescues cdc25-22 at restrictive temperature. The mts2 allele used to construct strains SP1212 and SP1213.
25	

The dephosphorylation of cdc2 on tyrosine 15 is a rate limiting step for the transition 30 of *S. pombe* cells from G2 into mitosis (Gould and Nurse, (1989) *Nature* 342:39-45). The tyrosine phosphorylation state of cdc2 is determined by a balance between the activities of the mitotic inhibitory tyrosine kinases wee1 and mik1 and the mitotic activating tyrosine phosphatases cdc25 and pyp3 (Russell and Nurse, (1986) *Cell* 45:145-153; Russell and Nurse, (1987) *Cell* 49:559-567; Lundgren et al., (1991) *Cell* 64:1111-1122; Dunphy and 35 Kumagai, (1991) *Cell* 67:189-196; Gautier et al., (1991) *Cell* 67:197-211; Millar et al., (1992) *EMBO J.* 11:4933-4941). Cdc25 and wee1 are the predominant activities (Lundgren et. al., (1991) *supra*; Millar et al., (1992) *supra*). To test for a genetic interaction between *pub1* and either *cdc25* or *wee1*, we constructed the strains outlined in Table 2. This table also contains a summary of the phenotypes observed for each strain. *Wee1-50* is a temperature 40 sensitive recessive loss of function allele of the nonessential gene *wee1* (Nurse, (1975) *Nature* 256:547-551; Nurse and Thuriaux, (1980) *Genetics* 96:627-637). At restrictive temperature (37°C) cells bearing a *wee1-50* allele undergo mitosis at a cell size significantly smaller than wild type. Loss of *wee1* function in a *pub1* disruption background is lethal. The apparent fragmentation of chromosomes, formation of anucleate cells and occasional 45 septation through the nucleus observed in the double mutant (SP 1209) at restrictive

temperature are thought to be the result of premature mitosis (Russell and Nurse, (1987) *supra*; Lundgren et al., (1991) *supra*). A similar mitotic lethality with *wee1-50* has been observed in several cases: in the absence of *mik1*; in the presence of the dominant activated *cdc2* allele *cdc2-3w*; in the presence of G2/M checkpoint mutants and in cells overproducing 5 *cdc25* (Lundgren et al., (1991) *supra*; Russell and Nurse, (1986) *supra*; Russell and Nurse, (1987) *supra*; Al-Khodairy and Carr, (1992) *EMBO J.* 11:1343-1350; Rowley et al., (1992) *EMBO J.* 13:335-1342; Enoch et al., (1992) *Gene & Dev.* 6:2035-2046; Walworth et al., (1993) *Nature* 363:369-371).

10 **Table 2**
Genetic Interactions

A.		20°C	25°C	30°C	37°C
15	<i>pub1::ura4</i>	+	+	+	+
	<i>wee1-50</i>	+	+	+	+
	<i>pub1::ura4, wee1-50</i>	+	+	+	- (l.m.)
	<i>cdc25-22</i>	+	+	+	- (<i>cdc</i> ⁻)
20	<i>pub1::ura4, cdc25-22</i>	-	+	+	- (<i>cdc</i> ⁻)
	<i>pub1::ura4, wee1-50, cdc25-22</i>	-	+	+	+

B.		+ thiamin	- thiamin
25	<i>pub1 ++ pREP41</i>	+	+
	<i>pub1 ++ pREP41wee1</i>	+	- (<i>cdc</i> ⁻)
	<i>pub1 ++ pREP41mik1</i>	+	- (<i>cdc</i> ⁻)
	<i>pub1::ura4 + pREP41</i>	+	+
	<i>pub1::ura4 + pREP41wee1</i>	+	+
	<i>pub1::ura4 + pREP41mik1</i>	+	+
30	<i>pub1 ::ura4 + pREP1</i>	+	+
	<i>pub1 ::ura4 + pREP1 wee1</i>	+	- (<i>cdc</i> ⁻)
	<i>pub1::ura4 + pREP1mik1</i>	+	- (<i>cdc</i> ⁻)

l.m. = lethal mitosis

35 *cdc*⁻ = cell cycle arrest with single nuclei and elongated cells. These cells arrest at the G2/M boundary with a 2N DNA content.

40 To determine if the *pub1::ura4 wee1-50* synthetic lethality requires *cdc25* function we constructed a *pub1::ura4 wee1-50 cdc25-22* triple mutant (SP 1211). *Cdc25-22* is a temperature sensitive, recessive, loss of function allele of *cdc25*. *Cdc25* is an essential gene whose loss results in cell-cycle arrest at the G2/M boundary with tyrosine phosphorylated *cdc2* and characteristically elongated cells (Russell and Nurse, (1986) *supra*; Gould and Nurse, (1989) *supra*; Lundgren et al., (1991) *supra*). Loss of *cdc25* function however can be rescued by the simultaneous loss of the antagonistic *wee1* function (Fantes, (1979) *Nature*

279:428-430). Thus, a *wee1-50 cdc25-22* double mutant is viable at the restrictive temperature of both single mutants. The *pub1::ura4 wee1-50 cdc25-22* triple mutant is also viable at restrictive temperature indicating that the mitotic lethality of the *pub1::ura4 wee1-50* double mutant requires *cdc25* function. This is not true of the *mikl::ura4 wee1-50 cdc25-22* triple mutant which undergoes lethal premature mitosis at restrictive temperature (Lundgren et al., (1991) *supra*). The viability of the *pub1::ura4 wee1-50 cdc25-22* triple mutant at restrictive temperature suggests that the lethal premature entry into mitosis by *pub1::ura4 wee1-50* is not the result of *pub1::ura4* dependent inhibition of *mikl*. The difference between the phenotypes of the two triple mutants can be understood as a simple case of epistasis. If a cell prematurely enters mitosis because it has lost both *wee1* and *mikl* function and thus cannot tyrosine phosphorylate *cdc2*, a dramatic reduction in the ability to tyrosine dephosphorylate *cdc2* will not rescue the cell. If, on the other hand, the tyrosine phosphatase activity of both *cdc25* and *pyp3* are simply overwhelming the ability of *mikl* to phosphorylate *cdc2*, loss of *cdc25* function could potentially rescue the cell. The lethal mitotic phenotype of the *pub1::ura4 wee1-50* double mutant and viability of the *pub1::ura4 wee1-50 cdc25-22* triple mutant suggests that the loss of *pub1* function activates either *cdc25* or *pyp3* or both.

IV. *Pub1* disruption increases tolerance of *wee1* and *mikl* overproduction

If disruption of *pub1* activates *cdc25* or *pyp3*, the disruption should act antagonistically to both *wee1* and *mikl*. To test this, we overproduced *wee1* and *mikl* in both *pub1+* (SP6) and *pub1::ura4* (SP 1208) cells. pREP41 is a *S. pombe* expression vector with a inducible *nmt* promoter (Maudrell, (1993) *Gene* 123:127-130). Expression from the *nmt* promoter is induced by starvation for thiamin. In the pREP41 plasmid the *nmt* promoter has been mutated to reduce the level of induction about 10 fold below that of the wild type *nmt* promoter. *Wee1* and *mikl* expressed from pREP41 plasmids will arrest the cell-cycle of wild type *S. pombe* at the G2/M boundary. pREP41 plasmids containing either *wee1*, *mikl* or no insert were introduced into either a *pub1+* (SP6) or a *pub1-* (SP 1208) strains and grown in the presence of thiamin. Transformants were then tested for their ability to form colonies in the absence of thiamin. A summary of the results can be seen in Table 2. *Pub1+* cells expressing either *wee1* or *mikl* from a pREP41 vector failed to form colonies in the absence of thiamin. The cells arrest at the G2/M boundary with a classic *cdc*-elongated cell phenotype. In contrast, *pub1-* cells expressing *wee1* or *mikl* from a pREP41 vector could readily form colonies in the absence of thiamin. Loss of *pub1* does not affect the level of either *wee1* or *mikl* expression in these strains as determined by Western blot analysis. Nor, does loss of *pub1* render *S. pombe* fully insensitive to overproduction of either *wee1* or *mikl*. *Pub1-* cells expressing either *wee1* or *mikl* from the pREP41 vector, divided at a cell size noticeably longer than vector controls. Furthermore, when either *wee1* or *mikl* is expressed

at higher levels using a wild type nmt promoter in a pREPI vector, *pub1*- cells cannot form colonies in the absence of thiamin (Maudrell, (1993) *supra*). These cells have the same terminal phenotype exhibited by *pub1*+ cells at lower levels of wee1 and mik1 expression. Thus, loss of *pub1* acts antagonistically to both wee1 and mik1 increasing the amount of these 5 kinases required to arrest the cell-cycle.

V. The pub1::ura4,cdc25-22 double mutant is cold sensitive

To further investigate the possibility of a genetic interaction between *pub1* and 10 *cdc25*, we constructed a *pub1::ura4 cdc25-22* double mutant (SP 1210). At the restrictive temperature of *cdc25-22*, this double mutant arrests at the G2/M boundary with the same terminal phenotype of a *cdc25-22* single mutant (SP628) - elongated cells with a 2N DNA content. Overexpression of *pyp3* will rescue a *cdc25-22* mutant at restrictive temperature 15 (Millar et al., (1992) *EMBO J.* 11:4944-4941). If the *pub1* disruption is activating *pyp3*, it is insufficient for such a rescue. The *cdc25-22* mutation can also be rescued by loss of *wee1* function. Thus, if *pub1::ura4* is inhibiting *wee1*, it is also insufficient to rescue the loss of *cdc25* function.

While either the *pub1::ura4* or the *cdc25-22* single mutants grow well at reduced 20 temperature, the *pub1::ura4 cdc25-22* double mutant is a cold sensitive synthetic lethal, unable to grow at 20°C. Interestingly, the terminal phenotype of this double mutant is not the *cdc*- elongated cell phenotype caused by loss of *cdc25* function nor the *wee* phenotype resulting from a significant increase in the level of active *cdc25*.

VI. Pub1 disruption elevates cdc25

25 To determine if *cdc25* is overexpressed in a *pub1* disruption background we examined the levels of *cdc25* mRNA and protein in both wild type (972) and *pub1::ura* (SP 1207) strains. Asynchronous cultures of both strains were grown to mid log at 30°C in YEA. Aliquots were removed from each culture for both Northern and Western blot analysis. 30 Loss of *pub* does not effect the steady state level of *cdc25* message. To determine the level of *cdc25* protein in these strains, lysates were prepared from mid log cultures grown at 30°C in YEA and subjected to Western blot analysis. Lysates prepared in an identical fashion from two additional cultures, a *cdc25* disruption (SP974) and a *cdc25* overproducer (SP1062) were used as controls for antibody specificity. (*Cdc2-3w* rescues loss of *cdc25* function in *S. pombe* (Russell and Nurse, (1987) *supra*.) The level of *cdc25* in asynchronously growing *pub1::ura4* cells is about 4 fold higher than wild type. The disruption of *pub1* post 35 transcriptionally increases the level of *cdc25* protein *in vivo*. Thus, *pub1* could either be inhibiting *cdc25* translation or enhancing its degradation.

VII. *Cdc25 is ubiquitinated in a pub1 dependent fashion*

5 The sequence similarity between *pub1* and the protein ubiquitin ligase E6-AP raised the possibility that *cdc25* may be ubiquitinated in *S. pombe* and that *pub1* may be involved. *Mts2* is a temperature sensitive mutant in the S4 subunit of the 26S proteosome in *S. pombe* (Gordon et al., (1993) *Nature* 366:355-357). The 26S proteosome is responsible for the 10 degradation of protein ubiquitin conjugates and these conjugates accumulate in a *mts2* mutant at restrictive temperature (Finley and Chau, (1991) *Annu. Rev. Cell Biol.* 7:25-69; Gordon et al., (1993) *supra*). To determine if *mts2* mutants accumulate ubiquitinated *cdc25* in a *pub1* dependent fashion we constructed the strains SP 1212 (*h^{-s} mts2 cdc25(HA)₃ leu1-32*) and SP 1213 (*h^{-s} pub1::ura4 mts2 cdc25(HA)₃ura4DI8 leu1-32*). These strains have 15 either wild type *pub1* (SP 1212) or the *pub1* disruption (SP 1213) present in an *mts2* mutant background. To ensure the specificity of the immunological detection of *cdc25*, both strains have the wild type *cdc25* gene replaced by a *cdc25* gene tagged with a triple tandem copy of the hemagglutinin epitope (HA). Cultures of both SP 1212 and SP 1213 were grown to early 20 log phase in YEA at 25°C. Both cultures were then shifted to restrictive temperature and aliquotes removed at the times indicated. Lysates prepared from these time points were analyzed by Western blot using the 12CA5 anti HA monoclonal antibody to detect *cdc25*. At both permissive and restrictive temperatures, *mts2* mutants accumulate a ladder of higher 25 molecular weight species of *cdc25*. Incubation at restrictive temperature appears to slightly increase the abundance of high molecular weight *cdc25* species, but otherwise have little effect. In contrast, in a *pub1* disruption background no accumulation of higher molecular weight species of *cdc25* is observed. If the exposure is extended for a prolonged period of time however, a faint ladder of *cdc25* higher molecular weight species can be seen in a *pub1* disruption background. We estimate that disruption of *pub1* reduces the accumulation of 30 *cdc25* higher molecular weight species at least 10 fold. No immunological crossreactivity was detected in congenic control strains lacking hemagglutinin epitope tagged *cdc25*.

To determine if the higher molecular weight *cdc25* species accumulated in an *mts2* mutant were the result of ubiquitination, we analyzed *cdc25* immunoprecipitates by Western blot for the presence of ubiquitin. Mid log cultures of both SP 1212 and SP 1213 were 35 incubated at restrictive temperature for 3 hours and lysates prepared. *Cdc25* was immunoprecipitated from each lysate. The immunoprecipitate from each strain was divided into two aliquotes, one analyzed by Western blot for the presence of *cdc25* and the other for the presence of ubiquitin. At restrictive temperature *mts2* mutants accumulate a ladder of higher molecular weight *cdc25* species in a *pub1* dependent fashion. These higher molecular weight forms of *cdc25* crossreact with an anti ubiquitin antibody indicating that they are

ubiquitinated forms of cdc25. The antigenicity of the cdc25 ubiquitin conjugates differs for anti HA and anti ubiquitin antibodies. The anti ubiquitin antibody preferentially recognizes the higher molecular weight forms of ubiquitinated cdc25. This is consistent with multiple ubiquitination - as the molecular weight of the species increases there are more ubiquitin molecules per molecule of cdc25. Both the higher molecular weight forms of cdc25 and the anti-ubiquitin crossreactive species are absent in a *pub1* deletion background indicating that the predominant mechanism for ubiquitination of cdc25 requires *pub1*.

VIII. Pub1 is thiol ubiquitinated in vivo

10

If *pub1* is functioning as an E6-AP like protein ubiquitin ligase, it should transfer ubiquitin from an E2 molecule to its target through a *pub1* thiol ubiquitinated intermediate (Scheffner et al., (1993) *Cell* 75:495-505; Scheffner et al., (1995) *Nature* 373:81-83). We designed a simple experiment to trap this putative intermediate *in vivo*. If the synthesis of new target molecules is blocked, the ubiquitin degradation system may degrade all accessible target molecules. In the absence of target molecules, the components of the ubiquitin cycle which employ ubiquitin thioester intermediates may accumulate in their ubiquitin charged intermediate form. The thiol ubiquitinated intermediate of interest could then be isolated by simple immunoprecipitation. The formation of new target molecules can be prevented by inhibition of translation with cycloheximide (Novak and Mitchison, (1987) *J. Cell Sci.* 87:323-325). Since ubiquitin is recycled, blocking translation should not deplete the cellular stores of ubiquitin (Finley and Chau, (1991) *supra*).

Pub1 tagged on the C-terminus with a single hemagglutinin epitope was cloned into the NdeI-BamHI sites of a pREPI forming pREPIpub1HA. This vector was introduced into a *pub1* deletion strain (SP 1208) in the presence of thiamin. Untagged *pub1* cloned into the same sites of pREPI (pREPIpub1) and introduced into the same parental strain was used as a control. Overexpression of *pub1* from a pREPI vector is lethal. The growth rate of SP 1208 containing either tagged or untagged *pub1* in a pREPI vector is unaffected for 12 hours after shifting the cells to minus thiamin media. Cells bearing either plasmid arrest 18 hours after induction. SP 1208 containing either pREPIpub1 or pREPIpub1HA was grown in minimal liquid media in the presence of thiamin and then shifted to minus thiamin media for 10 hours. At this point cycloheximide was added to the cultures and aliquots removed at the indicated times. The concentration of cycloheximide used was sufficient to reduce translation over 95% within 30 minutes (Novak and Mitchison, (1987) *supra*). Cell lysates were prepared from each time point in the presence of 1% LDS to prevent degradation and in the absence of any reducing agent to prevent displacement of the thioester bound ubiquitin. Gel samples from each time point were prepared in both the presence and absence of β mercaptoethanol. Samples prepared in the presence of β mercaptoethanol were subjected to electrophoresis in a standard Laemmeli gel at room temperature. Samples prepared in the

absence of β mercaptoethanol were subjected to electrophoresis on a modified Laemmeli gel at 4°C. Western blots of both gels were performed using the anti HA monoclonal antibody 12CA5 to detect *pub1*. There is a time dependent accumulation of two slower migrating forms of *pub1* in cycloheximide treated SP 1208 + pRE1pub1HA cells in the absence of 5 reducing agent. In the presence of reducing agents, both species are absent. The decrease in mobility exhibited by *pub1*-a could be accounted for by the addition of a single molecule of ubiquitin. *Pub1*-b migrates at the rate predicted for a molecule of approximately twice the molecular weight of *pub1*. Such a species could be the product of disulfide bond formation 10 between two *pub1* molecules or between *pub1* and a protein of approximately the same molecular weight. In the absence of a hemagglutinin tag, no *pub1* was detected.

If thiol ubiquitinated *pub1* accumulates in cycloheximide treated cells it should be possible to co-immunoprecipitate these two proteins in the absence of reducing agents. Logarithmically growing cultures of both SP 1208 + pRE1pub1 and SP 1208 + pRE1pub1HA were shifted to minus thiamine media for 10 hours to induce *pub1* 15 expression. Cycloheximide was then added. Lysates were prepared in the absence of reducing agents from both cultures immediately before and one hour after the addition of cycloheximide. The cells were lysed under strongly denaturing conditions (1% LDS) to disassociate non-covalent protein interactions and inhibit both isopeptidases and general proteolytic degradation activity (Haas et al., (1985) *J. Biol. Chem.* 260:4694-4703; Viersta et 20 al., (1985) *J. Biol. Chem.* 260:12015-12021). *Pub1* was immunoprecipitated from these lysates with the 12CA5 monoclonal antibody and the immunoprecipitates were divided into two aliquotes. One aliquote was incubated for 1 hour at 4°C in RIPA buffer containing 20 mM DTT and then washed twice with the same buffer. The other aliquote was treated identically with RIPA buffer lacking DTT. Both DTT treated and untreated 25 immunoprecipitates were analyzed by Western blot for the presence of *pub* and ubiquitin. One hour after treating the cells with cycloheximide, ubiquitin co-immunoprecipitates with *pub1*. *Pub1* and ubiquitin are both absent from the cycloheximide treated untagged *pub* control, indicating that the co-immunoprecipitation of ubiquitin with *pub1* is specific. The association between these two proteins is both DTT sensitive and dependent upon 30 cycloheximide treatment of the cells. The amino acid sequence of ubiquitin encoded by the UBI3 genes of *S. cerevisiae* and *S. pombe* are identical, both lack cysteine residues (Ozkaynak et al., (1987) *EMBO J.* 6:1429-1439; D. Conklin and D. Beach, unpublished results). Unless *S. pombe* contains another isoform of ubiquitin with a cysteine residue, these two proteins cannot be linked by a disulfide bond. In addition, the association of 35 ubiquitin with *pub1* was sensitive to 0.1M NaOH and refractile to 1M formic acid as are thioesters in general (Scheffner et al., 1995). Thus, the association of ubiquitin with *pub* is refractile to both 1% SDS and 1M formic acid, sensitive to both DTT and 0.1 M NaOH and coincident with the appearance of *pub1*-a, an approximately 5-10 kD DTT sensitive higher

molecular weight form of *pub1*. These observations suggest the presence of a thioester bond between *pub1* and ubiquitin *in vivo*.

IX. Cloning of human pub homologs

5 Human homologs of the fission yeast *pub1* gene were isolated by in order to investigate the role of ubiquitination in the regulation of *cdc25* in mammalian cells. The fission yeast *pub1* sequence was used to search DNA sequence databases to identify human sequences to be used as probes for the isolation of cDNA clones corresponding to s-*pub1*. The PCR primer probes 5'-GAAATGTTGAATCCATACTAT and 5'-
10 CCATATGCATTATGTTAACACAG were used to amplify the h-*pub1* sequence from a human keratinocyte cDNA library. Likewise, the PCR primers 5'-GACTTAGTCATCCAGTGGAG and 5'-CAAAACCAAGAGCATTCCACGG were used to amplify the h-*pub2* sequence from that same library. Altogether, five new human protein ubiquitin ligases, h-*pub1*-5, were isolated.

15 Three of the clones, h-*pub1*, h-*pub2* and h-*pub3* have been completely sequenced. These three genes were observed to have high homology to the yeast *pub1* gene.

In addition, we have observed in preliminary experiments that both h-*pub1* and h-*pub2* can become thiol-ubiquitinated *in vitro*.

20 *X. Complementation of s-pub1 disruptants with human h-pub1 protein*

The biological activity of the h-*pub1* gene was analyzed in a yeast complementation assay. Fission yeast strains in which *pub1* and *wee1* are simultaneously inactivated (*pub1::ura wee1-50*, described above) undergo pre-mature entry into mitosis. This is manifested by initiation of nuclear division at a small cell size, and is a lethal event. The 25 human *pub1* gene was expressed under control of the fission yeast *nmt1* promoter and transformed into this double mutant strain. These transformants (*pub1::ura wee1-50 h-pub1*) were then grown under selective conditions at either permissive (25°C) or non-permissive (36°C) conditions. The h-*pub1* protein was found to complement the loss of the fission yeast gene and to restore the cell size at mitosis to that of a wild-type cell. This 30 suggests that h-*pub1* is a biologically active, functional homolog of yeast *pub1*.

EXPERIMENTAL PROCEDURES:

Strains and Media:

35

All *S. pombe* strains were derived from the wild type strains originally described by Leupold (1970). All strains used in this study are listed in Table 1. *S. pombe* was grown in standard YE, YEA and PM media (Beach et al., (1985) *Nature* 300:706-709) containing additional leucine as described at 150µg/ml. Media referred to as thiamine (+) was

supplemented with 20 μ M thiamine. Standard *S. pombe* genetic procedures were followed (Glutz et al., (1974) *Handbook of Genetics 1*, R.C. King ed. New York Plenum Press pp 395-446).

5

Cloning Sequencing and Mapping pub1

An *S. pombe* cDNA library cloned into the NotI site of λ ZAP2 was immunologically screened with the anti-phosphotyrosine monoclonal antibody (UBI) (Lindberg et al., (1988) 10 *supra*; Lindberg and Pasquale, (1991) *supra*; Druker et al., (1989) *New Eng. J. Med.* 321:1383-1391). Two hundred and fifty thousand plaques were screened and two putative positive clones were identified which were capable of generating anti-phosphotyrosine 15 positive clones were identified which were capable of generating anti-phosphotyrosine crossreactive polypeptides upon induction in *E. coli* as determined by Western blot analysis. *Pub1* was one of these genes. The originally isolated *pub1* insert was used to rescreen the λ ZAP2 *S. pombe* cDNA library by hybridization to obtain a full length cDNA.

A series of unidirectional deletions of both the original *pub1* isolate and full length *pub1* cDNA were constructed in either pBluescript SK- or pBluescript KS- (Henikoff, (1987) 20 *Meth. Enzymol.* 155:156-165). Both strands of the cDNA clones were sequenced with a semiautomatic DNA sequencer (ABI 373A DNA sequencer). The predicted amino acid sequence was generated by an Intelligenetics program translation of the cDNA sequence. A blast search of Genbank was then performed to identify proteins with similar sequences.

To physically map the *pub1* gene we probed a collection of contiguous cosmid clones 25 spanning the *S. pombe* genome with the 2.8 kb *pub1* cDNA (Mizukami et al., (1993) *Cell* 73:121-132). Three overlapping cosmids, 323, 437 and 1187 hybridized to our probe indicating that *pub1* is on the right arm of chromosome 1 near the centromere.

Gene Disruption:

A 948bp SalI-NsiI fragment of the *pub1* cDNA was replaced with the 1.8 kb *ura4* 30 gene. The resulting 3.7 kb disrupted cDNA isolated as a NotI fragment was introduced the diploid strain SP826. Seventeen of the one hundred Ura⁺ transformants screened were unable to form single colonies in the presence of FOA indicating that the Ura⁺ phenotype was stable and consequently that the disrupted gene had been integrated. Seven of the ten Ura⁺ strains analyzed by Southern blot analysis carried both one copy of the *pub1* 35 disruption and one copy of the wild type gene. Colonies from one of these strains were screened by iodine staining for the ability to sporulate - a consequence of conversion of the mat locus from h+N/h+N to h90/h+N. Tetrads from this diploid were dissected and found produce four viable progeny.

Southern and Northern Analysis:

Genomic *S. pombe* DNA was isolated from strains 972 and SP 1207, digested with EcoRI and Southern blot analysis performed (Moreno et al., (1991) *Meth. Enzymol.* 194:795-823; Sambrook et al., (1989) *Molecular Cloning: A Laboratory Manual*, second ed.) . The 5 2.1 kb Eco RI fragment from the *pub1* cDNA was used as a probe.

Total *S. pombe* RNA was isolated according to Caligari (personal communication). Ten milliliter cultures of both 972 and SP 1207 were grown to mid log in YEA at 30°C and harvested. The cells were washed once with water and resuspended in a lysis buffer 10 composed of 100 µl TLSE (10 mM TrisHCl pH 7.5, 500 mM LiCl, 1 mM EDTA, 1% lithium lauryl sulfate) containing 1.7 mg/ml Heparin. One hundred microliters of phenol were added and the cells were lysed by vortexing for five minutes in the presence of glass 15 beads. Four hundred microliters of both TSLE and phenol were then added and the mixture was vortexed once again briefly. The aqueous phase was then extracted twice with phenol and once with phenol/chloroform/isoamyl alcohol. The RNA was then precipitated with 2.5 volumes of ethanol. A second ethanol precipitation was then performed to reduce salt levels. Northern blot analysis was performed (Sambrook. et al., (1989) *supra*). Full length *pub1*, *cdc25* and *ura4* 32 P labeled by random priming were used as probes as indicated in the text.

20 *Epitope Tagging pub1*

The *pub1* cDNA was used as a template for a PCR reaction which introduced an NdeI site at the initiating methionine and a SpeI site immediately before the translational stop of the *pub1*. The NdeI/SpeI *pub1* fragment was then cloned into the NdeI/SpeI sites of the 25 bacterial expression vector pETSC/HA.1 which contains a single hemagglutinin epitope bounded by an SpeI site on the 5 prime end and a stop codon followed by a BamH1 site on the 3 prime end. The resulting construct fused the C terminus of *pub1* to the 11 amino acid peptide TSYPYDVPDYA containing a single hemagglutinin epitope preceded by the amino acids TS. The N terminal NdeI/BamH1 fragment of this epitope tagged *pub1* was cloned 30 into the NdeI/BamH1 sites of the *S. pombe* expression vector pREPI and the C terminal BamH1 fragment was then cloned into the BamH 1 site of the resulting construct. The final construct pREPIpub1.HA consists of the *pub1* ORF with a C terminal 11 amino acid extension containing the HA epitope cloned into the NdeI/BamH1 sites of pREPI.

35 *Immunoprecipitation and immunoblotting of cdc25*

S. pombe extracts for immunoblotting of *cdc25* were prepared as previously described (Ducommun et al., (1990) *supra*). Cell pellets were resuspended in twice their volume of buffer I (50 mM Tris-HCl pH=8, 8 M urea, 1 mM EGTA, 5 mM EDTA, 0.1 mM

PMSF, 1 μ g/ml leupeptin, 10 μ g/ml soybean trypsin inhibitor, 1 μ g/ml aprotinin and 10 μ g/ml TPCK (tosyl phenylalanine chloromethyl ketone). The cells were lysed by vortexing in the presence of glass beads and the soluble proteins were recovered by centrifugation. Samples were immediately removed and boiled for 3 minutes in Laemmli sample buffer.

5 The protein concentration of the lysate was then determined by Bradford dye binding assay using γ globulin as a standard (Bradford, (1976) *Anal. Biochem.* 72:248-254). The proteins were separated electrophoretically on an 8% Laemmli gel and transferred to nitrocellulose (Schleicher and Schuel, 0.45 μ m) with a Millipore semi-dry transfer apparatus (Laemmli, (1970) *Nature* 277:680-685). All manipulations of the cdc25 blots were performed at room

10 temperature. The blots were blocked with TBST (10 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.05% Tween 20) containing 3% non fat dry milk and 10 mM sodium azide overnight.

The blot containing untagged cdc25 was then incubated in the blocking buffer containing a 1:500 dilution of an affinity purified anti-cdc25 polyclonal antibody B 1 for four hours (Ducommun et al., (1990) *supra*). After 5 ten minute washes in TBST, the blot

15 was incubated for 30 minutes in 16ng/ml peroxidase conjugated Affi Pure goat anti rabbit IgG Fc fragment specific (Jackson Immunoresearch) in TBST containing 3% milk. After four 10 minute washes in TBST followed by one ten minute wash in TBS (10 mM tris-HCl pH 7.5, 150 mM NaCl), cdc25 was detected by ECL (Amersham).

Blots containing hemagglutinin tagged cdc25 were incubated for 2 hours in the

20 blocking buffer containing 100 ng/ml 12CA5 anti-HA monoclonal antibody (Boehringer and Mannheim). After 5 ten minute washes in TBST, the blots were incubated for 1 hour in 100 ng/ml peroxidase conjugated Affi Pure goat anti mouse IgG Fc fragment specific (Jackson Immunoresearch) in TBST containing 3% milk. After four ten minute washes in TBST followed by one wash in TBS, cdc25 was detected by ECL (Amersham).

25 To immunoprecipitate cdc25 we modified the previously described protocol (Ducommun et al., (1990) *supra*). Cell pellets were resuspended in 4 volumes of ice cold buffer 2 (25 mM Tris-HCl pH 8.0, 60 mM β -glycerol phosphate, 15 mM para-nitrophenylphosphate, 0.1 mM orthovanadate and 0.1 % Triton X 100) containing the same protease inhibitors as in buffer I. The cells were lysed by vortexing in the presence of glass

30 beads at 4°C and the extracts immediately made 1% SDS and boiled for 3 minutes. The extracts were then diluted 10 fold in RIPA buffer lacking SDS and soluble proteins recovered by centrifugation at 10,000g for 15 minutes at 4°C. The protein concentration was then determined by Bradford assay (Bradford, (1976) *supra*). The lysates were preincubated with protein A agarose (Pierce) for 30 minutes and centrifuged at 10,000g for 10 minutes

35 both at 4°C. Affinity purified B 1 anti-cdc25 polyclonal antibody was added to the supernatant and the mixture was incubated overnight at 4°C on a rotator. Non specific precipitated proteins were removed by centrifugation at 4°C for 5 minutes at 10,000g. The supernatant was incubated with protein A agarose for 30 minutes at 4°C and the immunoprecipitates collected by low speed centrifugation. The immunoprecipitates were

then washed five times with RIPA buffer after which the beads were resuspended in Laemmli sample buffer and boiled for 3 minutes. The supernatant was subjected to electrophoresis in an 8% Laemmli gel. Western blot analysis was subsequently performed to test for the presence of both ubiquitinated and hemagglutinin tagged cdc25.

5

Immunoblot detection of ubiquitin

The same protocol was used to detect both free ubiquitin and ubiquitinated cdc25. The gel of interest was transferred by the semi dry Milliblot-SDE system (Millipore) to a sheet of 0.2 μ m nitrocellulose (Schleicher and Schuel) which has been hydrated overnight in distilled water. After the transfer the nitrocellulose is incubated in distilled water at 100°C for 20 minutes (Swerdlow et al., (1986) *Anal. Biochem.* 156:147-153). All subsequent manipulations were done at room temperature. The blots were then blocked by overnight incubation with 3% BSA in TBST containing 10 mM sodium azide. The blots were incubated for 1 hour in blocking buffer containing an anti ubiquitin polyclonal antibody (Sigma) and subsequently washed five times for 10 minutes in TBST. The blots were then incubated with affinity purified HRP conjugated goat anti rabbit Fc (Jackson Immunoresearch) in TBST containing 3% BSA for 30 minutes. The blots were washed four times for ten minutes in TBST and then once in TBS. Ubiquitin was detected by ECL.

20

Immunoblotting and immunoprecipitation of thioester ubiquitinated pub1

Strain SP 1208 bearing either pREPIpub1HA or pREPIpub1 was grown in minimal media lacking thiamin for 10 hours to a density of 10⁷/ml after which cycloheximide was added to a final concentration of 100 μ g/ml. Immediately before and 15, 30, 60, and 120 minutes after the addition of cycloheximide 50 ml aliquotes were removed from the culture, the cells harvested by centrifugation, washed once with water and the cell pellets kept in a dry ice/ ethanol bath until the end of the time course. The cell pellets were then thawed and resuspended in 3 volumes of cold lysis buffer (50 mM HEPES-NaOH pH 7, 1% lithium lauryl sulfate, 150 mM NaCl, 10 mM iodoacetamide, 5 mM EDTA, 1 mM EGTA, 0.1 mM PMSF, 10 μ g/ml leupeptin, 10 μ g/ml soybean trypsin inhibitor, 1 μ g/ml aprotinin and 10 μ g/ml TPCK) (Hershko et al., (1982) *J. Biol. Chem.* 257:13964-13970; Haas et al., (1985) *supra*; Viestra et al., (1985) *supra*). The cells were lysed by vortexing in the presence of glass beads and the soluble proteins recovered by centrifugation at 10,000g for 15 minutes at 4°C. The protein concentration was determined by Bradford assay (Bradford, (1976) *supra*). Two gel samples of identical protein concentration were prepared from each time point one using standard Laemmli sample buffer and the other a modified Laemmli sample buffer which lacked β -mercaptoethanol and had LDS in place of SDS. The standard Laemmli samples were subjected to electrophoresis in an 8% Laemmli gel at room temperature. The

other samples were subjected to electrophoresis at 4°C in a modified Laemmli gel in which LDS is substituted for SDS (McGrath et al., (1991) *EMBO J.* 10:227-236). Both gels were transferred to nitrocellulose and subjected to Western blot analysis with the 12CA5 anti-HA monoclonal antibody as described above.

5 To immunoprecipitate thioester ubiquitinated pub1, strain SP 1208 containing pREPIpub1HA or pREPIpub1 was grown in minimal media lacking thiamin at 30°C for 10 hours to a density of 10⁷/ml. The culture was then made 100µg/ml cycloheximide and incubated for an additional hour at 30°C. Aliquots were removed immediately before and 1 hour after the addition of cycloheximide. The cells were harvested washed once with 10 distilled water and kept in a dry ice/ethanol bath until lysis. Lysates were prepared in buffer 2 as described above. The lysates were diluted ten fold with RIPA buffer containing the same protease inhibitors as buffer 2 and lacking SDS. The soluble proteins were recovered by centrifugation and the protein concentration determined by Bradford assay. The volume of lysates from each sample containing five mg of soluble protein were pre-cleared with 15 protein A agarose (Pierce) and then incubated with 12CA5 anti HA monoclonal antibody for 1 hour at 4°C on a rotator. The samples were then centrifuged at 4°C for 10 minutes at 10,000g to remove precipitated proteins and the supernatants incubated with protein A agarose for 30 minutes at 4°C on a rotator. The immunoprecipitates were collected by low speed centrifugation and washed 5 times with a modified RIPA buffer containing the same 20 protease inhibitors as buffer 2. The immunoprecipitates were then split into two equal 25 aliquotes. One aliquote was incubated for 1 hour at 4°C in RIPA buffer containing both protease inhibitors present in buffer I and 20 mM DTT and then washed twice the same buffer. The other aliquote was treated identically with the same buffer lacking DTT. The beads were resuspended in Laemmli sample buffer and boiled for 3 minutes. Samples from both aliquotes were run on both an 8% gel to detect pub1 and an 18% gel to detect free ubiquitin. *Pub1* and ubiquitin Westerns were performed as described above.

Miscellaneous

30 Dapi (4,6 diamidino-2-phenylindole) staining of *S. pombe* cells was done according to (Moreno et. al., (1991) *supra*).

Pub1 was transcribed and translated *in vitro* using the TNT T7 coupled reticulocyte lysate system (Promega) in the presence of ³⁵S Express (NEN).

All of the above-cited references and publications are hereby incorporated by reference.

5

Equivalents

Those skilled in the art will recognize, or be able to ascertain using no more than routine experimentation, many equivalents to the specific embodiments of the invention described herein. Such equivalents are intended to be encompassed by the following claims.

SEQUENCE LISTING

5 (1) GENERAL INFORMATION:

(i) APPLICANT:

- (A) NAME: Cold Spring Harbor Laboratory
- (B) STREET: One Bungtown Road
- (C) CITY: Cold Spring Harbor
- (D) STATE: NY
- (E) COUNTRY: USA
- (F) POSTAL CODE (ZIP): 11724

15 (ii) TITLE OF INVENTION: Ubiquitin Ligases, and Uses Related Thereto

(iii) NUMBER OF SEQUENCES: 8

(iv) COMPUTER READABLE FORM:

- (A) MEDIUM TYPE: Floppy disk
- (B) COMPUTER: IBM PC compatible
- (C) OPERATING SYSTEM: PC-DOS/MS-DOS
- (D) SOFTWARE: AscII(text)

25 (vi) PRIOR APPLICATION DATA:

- (A) APPLICATION NUMBER: US 08/539,205
- (B) FILING DATE: 04-OCT-1995

30 (2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 2247 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: both
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

40

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 1..2244

45

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

ATG TCT AAC CCC GGA GGC CGG AGG AAC GGG CCC GTC AAG CTG CGC CTG	48
Met Ser Asn Pro Gly Gly Arg Arg Asn Gly Pro Val Lys Leu Arg Leu	

50

1	5	10	15
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ACA GTA CTC TGT GCA AAA AAC CTG GTG AAA AAG GAT TTT TTC CGA CTT	96
Thr Val Leu Cys Ala Lys Asn Leu Val Lys Lys Asp Phe Phe Arg Leu	

20	25	30
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55

CCT GAT CCA TTT GCT AAG GTG GTG GAT GGA TCT GGG CAA TGC CAT	144
Pro Asp Pro Phe Ala Lys Val Val Asp Gly Ser Gly Gln Cys His	

35	40	45
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5	TCT ACA GAT ACT GTG AAG AAT ACG CTT GAT CCA AAG TGG AAT CAG CAT Ser Thr Asp Thr Val Lys Asn Thr Leu Asp Pro Lys Trp Asn Gln His 50 55 60	192
10	TAT GAC CTG TAT ATT GGA AAG TCT GAT TCA GTT ACG ATC AGT GTA TGG Tyr Asp Leu Tyr Ile Gly Lys Ser Asp Ser Val Thr Ile Ser Val Trp 65 70 75 80	240
15	AAT CAC AAG AAG ATC CAT AAG AAA CAA GGT GCT GGA TTT CTC GGT TGT Asn His Lys Ile His Lys Gln Gly Ala Gly Phe Leu Gly Cys 85 90 95	288
20	GTT CGT CTT CTT TCC AAT GCC ATC AAC CGG CTC AAA GAC ACT GGT TAT Val Arg Leu Leu Ser Asn Ala Ile Asn Arg Leu Lys Asp Thr Gly Tyr 100 105 110	336
25	CAG AGG TTG GAT TTA TGC AAA CTC GGG CCA AAT GAC AAT GAT ACA GTT Gln Arg Leu Asp Leu Cys Lys Leu Gly Pro Asn Asp Asn Asp Thr Val 115 120 125	384
30	AGA GGA CAG ATA GTA GTA AGT CTT CAG TCC AGA GAC CGA ATA GGC ACA Arg Gly Gln Ile Val Val Ser Leu Gln Ser Arg Asp Arg Ile Gly Thr 130 135 140	432
35	GGA GGA CAA GTT GTG GAC TGC AGT CGT TTA TTT GAT AAC GAT TTA CCA Gly Gly Gln Val Val Asp Cys Ser Arg Leu Phe Asp Asn Asp Leu Pro 145 150 155 160	480
40	GAC GGC TGG GAA GAA AGG AGA ACC GCC TCT GGA AGA ATC CAG TAT CTA Asp Gly Trp Glu Glu Arg Arg Thr Ala Ser Gly Arg Ile Gln Tyr Leu 165 170 175	528
45	AAC CAT ATA ACA AGA ACT ACG CAA TGG GAG CGC CCA ACA CGA CCG GCA Asn His Ile Thr Arg Thr Gln Trp Glu Arg Pro Thr Arg Pro Ala 180 185 190	576
50	TCC GAA TAT TCT AGC CCT GGC AGA CCT CTT AGC TGC TTT GTT GAT GAG Ser Glu Tyr Ser Ser Pro Gly Arg Pro Leu Ser Cys Phe Val Asp Glu 195 200 205	624
55	AAC ACT CCA ATT AGT GGA ACA AAT GGT GCA ACA TGT GGA CAG TCT TCA Asn Thr Pro Ile Ser Gly Thr Asn Gly Ala Thr Cys Gly Gln Ser Ser 210 215 220	672
60	GAT CCC AGG CTG GCA GAG AGG AGA GTC AGG TCA CAA CGA CAT AGA AAT Asp Pro Arg Leu Ala Glu Arg Arg Val Arg Ser Gln Arg His Arg Asn 225 230 235 240	720
65	TAC ATG AGC AGA ACA CAT TTA CAT ACT CCT CCA GAC CTA CCA GAA GGC Tyr Met Ser Arg Thr His Leu His Thr Pro Pro Asp Leu Pro Glu Gly 245 250 255	768
70	TAT GAA CAG AGG ACA ACG CAA CAA GGC CAG GTG TAT TTC TTA CAT ACA Tyr Glu Gln Arg Thr Thr Gln Gln Gly Gln Val Tyr Phe Leu His Thr 260 265 270	816
75	CAG ACT GGT GTG AGC ACA TGG CAT GAT CCA AGA GTG CCC AGG GAT CTT Gln Thr Gly Val Ser Thr Trp His Asp Pro Arg Val Pro Arg Asp Leu	864

	275	280	285	
5	290	295	300	912
10	305	310	315	960
15	325	330	335	1008
20	340	345	350	1056
25	355	360	365	1104
30	370	375	380	1152
35	385	390	395	1200
40	405	410	415	1248
45	420	425	430	1296
50	435	440	445	1344
55	450	455	460	1392
60	465	470	475	1440
65	485	490	495	1488
70	500	505	510	1536
	CTT GGG AAG TCA ATT ACC TTG GAT GAC ATG GAG TTA GTA GAT CCG GAT			1584

Leu	Gly	Lys	Ser	Ile	Thr	Leu	Asp	Asp	Met	Glu	Leu	Val	Asp	Pro	Asp		
515						520				525							
5	CTT	CAC	AAC	AGT	TTA	GTG	TGG	ATA	CTT	GAG	AAT	GAT	ATT	ACA	GGT	GTT	1632
	Leu	His	Asn	Ser	Leu	Val	Trp	Ile	Leu	Glu	Asn	Asp	Ile	Thr	Gly	Val	
	530					535				540							
10	TTG	GAC	CAT	ACC	TTC	TGT	GTT	GAA	CAT	AAT	GCA	TAT	GGT	GAA	ATT	ATT	1680
	Leu	Asp	His	Thr	Phe	Cys	Val	Glu	His	Asn	Ala	Tyr	Gly	Glu	Ile	Ile	
	545					550				555					560		
15	CAG	CAT	GAA	CTT	AAA	CCA	AAT	GGC	AAA	AGT	ATC	CCT	GTT	AAT	GAA	GAA	1728
	Gln	His	Glu	Leu	Lys	Pro	Asn	Gly	Lys	Ser	Ile	Pro	Val	Asn	Glu	Glu	
	565					570				575							
20	AAT	AAA	AAA	GAA	TAT	GTC	AGG	CTC	TAT	GTG	AAC	TGG	AGA	TTT	TTA	CGG	1776
	Asn	Lys	Lys	Glu	Tyr	Val	Arg	Leu	Tyr	Val	Asn	Trp	Arg	Phe	Leu	Arg	
	580					585				590							
25	GGC	ATT	GAG	GCT	CAA	TTC	TTG	GCT	CTG	CAG	AAA	GGA	TTT	AAT	GAA	GTA	1824
	Gly	Ile	Glu	Ala	Gln	Phe	Leu	Ala	Leu	Gln	Lys	Gly	Phe	Asn	Glu	Val	
	595					600				605							
30	ATT	CCA	CAA	CAT	CTG	CTG	AAG	ACA	TTT	GAT	GAG	AAG	GAG	TTA	GAG	CTC	1872
	Ile	Pro	Gln	His	Leu	Leu	Lys	Thr	Phe	Asp	Glu	Lys	Glu	Leu	Glu	Leu	
	610					615				620							
35	ATT	ATT	TGT	GGA	CTT	GGA	AAG	ATA	GAT	GTT	AAT	GAC	TGG	AAG	GTA	AAC	1920
	Ile	Ile	Cys	Gly	Leu	Gly	Lys	Ile	Asp	Val	Asn	Asp	Trp	Lys	Val	Asn	
	625					630				635					640		
40	ACC	CGG	TTA	AAA	CAC	TGT	ACA	CCA	GAC	AGC	AAC	ATT	GTC	AAA	TGG	TTC	1968
	Thr	Arg	Leu	Lys	His	Cys	Thr	Pro	Asp	Ser	Asn	Ile	Val	Lys	Trp	Phe	
	645					650				655							
45	TGG	AAA	GCT	GAG	TTT	TTT	GAT	GAA	GAG	CGA	CGA	GCA	AGA	TTG	CTT	2016	
	Trp	Lys	Ala	Val	Glu	Phe	Asp	Glu	Glu	Arg	Arg	Ala	Arg	Leu	Leu		
	660					665				670							
50	CAG	TTT	GTC	ACA	GGA	TCC	TCT	CGA	GTG	CCT	CTG	CAG	GGC	TTC	AAA	GCA	2064
	Gln	Phe	Val	Thr	Gly	Ser	Ser	Arg	Val	Pro	Leu	Gln	Gly	Phe	Lys	Ala	
	675					680				685							
55	TTG	CAA	GGT	GCT	GCA	GGC	CCG	AGA	CTC	TTT	ACC	ATA	CAC	CAG	ATT	GAT	2112
	Leu	Gln	Gly	Ala	Ala	Gly	Pro	Arg	Leu	Phe	Thr	Ile	His	Gln	Ile	Asp	
	690					695				700							
60	GCC	TGC	ACT	AAC	AAC	CTG	CCG	AAA	GCC	CAC	ACT	TGC	TTC	AAT	CGA	ATA	2160
	Ala	Cys	Thr	Asn	Asn	Leu	Pro	Lys	Ala	His	Thr	Cys	Phe	Asn	Arg	Ile	
	705					710				715					720		
65	GAC	ATT	CCA	CCC	TAT	GAA	AGC	TAT	GAA	AAG	CTA	TAT	GAA	AAG	CTG	CTA	2208
	Asp	Ile	Pro	Pro	Tyr	Glu	Ser	Tyr	Glu	Lys	Leu	Tyr	Glu	Lys	Leu	Leu	
	725					730				735							
70	ACA	GCC	ATT	GAA	GAA	ACA	TGT	GGA	TTT	GCT	GTG	GAA	TGA				2247
	Thr	Ala	Ile	Glu	Glu	Thr	Cys	Gly	Phe	Ala	Val	Glu					
	740					745											

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

5 (A) LENGTH: 748 amino acids
 (B) TYPE: amino acid
 (C) TOPOLOGY: linear

10 (ii) MOLECULE TYPE: protein

15 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

Met	Ser	Asn	Pro	Gly	Gly	Arg	Arg	Asn	Gly	Pro	Val	Lys	Leu	Arg	Leu	
1				5					10				15			
15	Thr	Val	Leu	Cys	Ala	Lys	Asn	Leu	Val	Lys	Lys	Asp	Phe	Phe	Arg	Leu
				20				25				30				
20	Pro	Asp	Pro	Phe	Ala	Lys	Val	Val	Val	Asp	Gly	Ser	Gly	Gln	Cys	His
				35				40				45				
25	Ser	Thr	Asp	Thr	Val	Lys	Asn	Thr	Leu	Asp	Pro	Lys	Trp	Asn	Gln	His
				50				55				60				
30	Tyr	Asp	Leu	Tyr	Ile	Gly	Lys	Ser	Asp	Ser	Val	Thr	Ile	Ser	Val	Trp
				65				70			75		80			
35	Asn	His	Lys	Lys	Ile	His	Lys	Lys	Gln	Gly	Ala	Gly	Phe	Leu	Gly	Cys
				85				90				95				
40	Val	Arg	Leu	Leu	Ser	Asn	Ala	Ile	Asn	Arg	Leu	Lys	Asp	Thr	Gly	Tyr
				100				105				110				
45	Gln	Arg	Leu	Asp	Leu	Cys	Lys	Leu	Gly	Pro	Asn	Asp	Asn	Asp	Thr	Val
				115				120				125				
50	Arg	Gly	Gln	Ile	Val	Val	Ser	Leu	Gln	Ser	Arg	Asp	Arg	Ile	Gly	Thr
				130				135				140				
55	Gly	Gly	Gln	Val	Val	Asp	Cys	Ser	Arg	Leu	Phe	Asp	Asn	Asp	Leu	Pro
				145				150			155		160			
60	Asp	Gly	Trp	Glu	Glu	Arg	Arg	Thr	Ala	Ser	Gly	Arg	Ile	Gln	Tyr	Leu
				165				170				175				
65	Asn	His	Ile	Thr	Arg	Thr	Thr	Gln	Trp	Glu	Arg	Pro	Thr	Arg	Pro	Ala
				180				185				190				
70	Ser	Glu	Tyr	Ser	Ser	Pro	Gly	Arg	Pro	Leu	Ser	Cys	Phe	Val	Asp	Glu
				195				200				205				
75	Asn	Thr	Pro	Ile	Ser	Gly	Thr	Asn	Gly	Ala	Thr	Cys	Gly	Gln	Ser	Ser
				210				215				220				
80	Asp	Pro	Arg	Leu	Ala	Glu	Arg	Arg	Val	Arg	Ser	Gln	Arg	His	Arg	Asn
				225				230			235		240			
85	Tyr	Met	Ser	Arg	Thr	His	Leu	His	Thr	Pro	Pro	Asp	Leu	Pro	Glu	Gly
				245				250				255				

Tyr Glu Gln Arg Thr Thr Gln Gln Gly Gln Val Tyr Phe Leu His Thr
 260 265 270

5 Gln Thr Gly Val Ser Thr Trp His Asp Pro Arg Val Pro Arg Asp Leu
 275 280 285

Ser Asn Ile Asn Cys Glu Glu Leu Gly Pro Leu Pro Pro Gly Trp Glu
 290 295 300

10 Ile Arg Asn Thr Ala Thr Gly Arg Val Tyr Phe Val Asp His Asn Asn
 305 310 315 320

Arg Thr Thr Gln Phe Thr Asp Pro Arg Leu Ser Ala Asn Leu His Leu
 15 325 330 335

Val Leu Asn Arg Gln Asn Gln Leu Lys Asp Gln Gln Gln Gln Val
 340 345 350

20 Val Ser Leu Cys Pro Asp Asp Thr Glu Cys Leu Thr Val Pro Arg Tyr
 355 360 365

Lys Arg Asp Leu Val Gln Lys Leu Lys Ile Leu Arg Gln Glu Leu Ser
 370 375 380

25 Gln Gln Gln Pro Gln Ala Gly His Cys Arg Ile Glu Val Ser Arg Glu
 385 390 395 400

Glu Ile Phe Glu Glu Ser Tyr Arg Gln Val Met Lys Met Arg Pro Lys
 30 405 410 415

Asp Leu Trp Lys Arg Leu Met Ile Lys Phe Arg Gly Glu Glu Gly Leu
 420 425 430

35 Asp Tyr Gly Gly Val Ala Arg Glu Trp Leu Tyr Leu Leu Ser His Glu
 435 440 445

Met Leu Asn Pro Tyr Tyr Gly Leu Phe Gln Tyr Ser Arg Asp Asp Ile
 450 455 460

40 Tyr Thr Leu Gln Ile Asn Pro Asp Ser Ala Val Asn Pro Glu His Leu
 465 470 475 480

Ser Tyr Phe His Phe Val Gly Arg Ile Met Gly Met Ala Val Phe His
 485 490 495

Gly His Tyr Ile Asp Gly Gly Phe Thr Leu Pro Phe Tyr Lys Gln Leu
 500 505 510

50 Leu Gly Lys Ser Ile Thr Leu Asp Asp Met Glu Leu Val Asp Pro Asp
 515 520 525

Leu His Asn Ser Leu Val Trp Ile Leu Glu Asn Asp Ile Thr Gly Val
 530 535 540

55 Leu Asp His Thr Phe Cys Val Glu His Asn Ala Tyr Gly Glu Ile Ile
 545 550 555 560

Gln His Glu Leu Lys Pro Asn Gly Lys Ser Ile Pro Val Asn Glu Glu

565 570 575

Asn Lys Lys Glu Tyr Val Arg Leu Tyr Val Asn Trp Arg Phe Leu Arg
 580 585 590

5

Gly Ile Glu Ala Gln Phe Leu Ala Leu Gln Lys Gly Phe Asn Glu Val
 595 600 605

10 Ile Pro Gln His Leu Leu Lys Thr Phe Asp Glu Lys Glu Leu Glu Leu
 610 615 620

Ile Ile Cys Gly Leu Gly Lys Ile Asp Val Asn Asp Trp Lys Val Asn
 625 630 635 640

15 Thr Arg Leu Lys His Cys Thr Pro Asp Ser Asn Ile Val Lys Trp Phe
 645 650 655

Trp Lys Ala Val Glu Phe Phe Asp Glu Glu Arg Arg Ala Arg Leu Leu
 660 665 670

20

Gln Phe Val Thr Gly Ser Ser Arg Val Pro Leu Gln Gly Phe Lys Ala
 675 680 685

25

Leu Gln Gly Ala Ala Gly Pro Arg Leu Phe Thr Ile His Gln Ile Asp
 690 695 700

Ala Cys Thr Asn Asn Leu Pro Lys Ala His Thr Cys Phe Asn Arg Ile
 705 710 715 720

30

Asp Ile Pro Pro Tyr Glu Ser Tyr Glu Lys Leu Tyr Glu Lys Leu Leu
 725 730 735

35

Thr Ala Ile Glu Glu Thr Cys Gly Phe Ala Val Glu
 740 745

(2) INFORMATION FOR SEQ ID NO:3:

40

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 2848 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: both
- (D) TOPOLOGY: linear

45

(ii) MOLECULE TYPE: cDNA

50

(ix) FEATURE:

- (A) NAME/KEY: CDS
- (B) LOCATION: 238..2535

55

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

AACGACGGAT TTCAAAATTG TCGCCTGAAA GACGAATTG ATTAAGGATT CCTTTTGTAG 60
 TTCTTTTTTA TTTATTCGTT CCCGAAAAAA GCGTTTTGT ATCAAATAGA AAGGATAAAA 120
 CGGCCTTAAT TGAAAGTTTT TTTTGCAAGC ACAAACTTT GACTTTCTTT GGGAAATTGG 180

CTTTTTTTTA TTATCCAAAA GCAACGCTGG TTTGAAATTT TACCATCTTT TCCCCCT	237
5 ATG TCA AAC TCA GCT CAA TCT CGT CGA ATT CGA GTA ATA ATT GTT GCT Met Ser Asn Ser Ala Gln Ser Arg Arg Ile Arg Val Ile Ile Val Ala 1 5 10 15	285
10 GCT GAT GGC CTT TAC AAA CGA GAT GTG TTT CGT TTT CCT GAC CCG TTT Ala Asp Gly Leu Tyr Lys Arg Asp Val Phe Arg Phe Pro Asp Pro Phe 20 25 30	333
15 GCG GTA CTA ACA GTG GAT GGT GAG CAA ACA CAT ACC ACA ACT GCT ATC Ala Val Leu Thr Val Asp Gly Glu Gln Thr His Thr Thr Ala Ile 35 40 45	381
20 AAA AAG ACG TTA AAC CCT TAT TGG AAT GAG ACT TTT GAA GTT AAT GTT Lys Lys Thr Leu Asn Pro Tyr Trp Asn Glu Thr Phe Glu Val Asn Val 50 55 60	429
25 ACA GAT AAT AGC ACC ATT GCG ATT CAG GTG TTC GAT CAG AAA AAA TTT Thr Asp Asn Ser Thr Ile Ala Ile Gln Val Phe Asp Gln Lys Lys Phe 65 70 75 80	477
30 AAG AAA AAA GGC CAA GGC TTT CTA GGA GTG ATA AAT CTT CGT GTT GGA Lys Lys Gly Gln Gly Phe Leu Gly Val Ile Asn Leu Arg Val Gly 85 90 95	525
35 GAT GTG CTC GAT CTC GCC ATT GGA GGT GAT GAA ATG TTG ATT TGC GAT Asp Val Leu Asp Leu Ala Ile Gly Gly Asp Glu Met Leu Ile Cys Asp 100 105 110	573
40 TTG AAA AAG TTA AAT GAG AAT ACA GTA GTT CAT GGC AAG ATA ATC ATA Leu Lys Leu Asn Glu Asn Thr Val Val His Gly Lys Ile Ile Ile 115 120 125	621
45 AAC TTA TCA ACG ACT GCG CAG TTA ACC TTA CAG GTT CCA TCC AGT GCA Asn Leu Ser Thr Thr Ala Gln Leu Thr Leu Gln Val Pro Ser Ser Ala 130 135 140	669
50 GCA TCA GGT GCA CGT ACC CAA CGT ACT AGC ATT ACC AAT GAC CCG CAA Ala Ser Gly Ala Arg Thr Gln Arg Thr Ser Ile Thr Asn Asp Pro Gln 145 150 155 160	717
55 AGC TCA AAA AGT AGC AGT GTG TCT CGT AAC CCT GCT TCC TCT CGT GCT Ser Ser Lys Ser Ser Val Ser Arg Asn Pro Ala Ser Ser Arg Ala 165 170 175	765
50 GGT TCA CCT ACC CGC GAC AAC GCA CCT GCT GCT TCC CCT GCT AGT TCA Gly Ser Pro Thr Arg Asp Asn Ala Pro Ala Ala Ser Pro Ala Ser Ser 180 185 190	813
55 GAA CCT CGC ACA TTT AGT TCA TTT GAA GAT CAA TAT GGG CGC CTT CCT Glu Pro Arg Thr Phe Ser Ser Phe Glu Asp Gln Tyr Gly Arg Leu Pro 195 200 205	861
55 CCT GGA TGG GAA AGA TGT ACC GAT AAT CTT GGC CGA ACT TAT TAT GTA Pro Gly Trp Glu Arg Cys Thr Asp Asn Leu Gly Arg Thr Tyr Tyr Val 210 215 220	909

GAT CAT AAT ACA AGA AGC ACA ACG TGG ATT CGC CCC AAC CTA AGT TCT	957
Asp His Asn Thr Arg Ser Thr Thr Trp Ile Arg Pro Asn Leu Ser Ser	
225 230 235 240	
5 GTT GCC GGA GCA GCC GCA GCC GAA TTG CAT AGT AGT GCA TCG TCT GCG	1005
Val Ala Gly Ala Ala Ala Glu Leu His Ser Ser Ala Ser Ser Ala	
245 250 255	
10 AAT GTT ACC GAA GGT GTT CAA CCT TCC TCT AGC AAT GCA GCT CGT CGT	1053
Asn Val Thr Glu Gly Val Gln Pro Ser Ser Asn Ala Ala Arg Arg	
260 265 270	
15 ACA GAA GCT AGT GTT TTG ACC TCT AAC GCT ACT ACT GCT GGT TCA GGA	1101
Thr Glu Ala Ser Val Leu Thr Ser Asn Ala Thr Ala Gly Ser Gly	
275 280 285	
20 GAG CTT CTA CCG GGA TGG GAG CAA AGG TAT ACA CCA GAG GGT CGA CCC	1149
Glu Leu Leu Pro Gly Trp Glu Gln Arg Tyr Thr Pro Glu Gly Arg Pro	
290 295 300	
25 TAC TTT GTG GAT CAT AAT ACG CGA ACT ACT ACT TGG GTG GAT CCG CGC	1197
Tyr Phe Val Asp His Asn Thr Arg Thr Thr Trp Val Asp Pro Arg	
305 310 315 320	
30 CGT CAA CAG TAC ATT CGT TCC TAT GGC GGT CCT AAT AAT GCT ACT ATT	1245
Arg Gln Gln Tyr Ile Arg Ser Tyr Gly Gly Pro Asn Asn Ala Thr Ile	
325 330 335	
35 CAG CAA CAA CCT GTC TCT CAA CTT GGT CCT TTG CCA AGT GGT TGG GAA	1293
Gln Gln Gln Pro Val Ser Gln Leu Gly Pro Leu Pro Ser Gly Trp Glu	
340 345 350	
40 ATG CGT CTT ACC AAT ACT GCT CGT GTA TAT TTT GTT GAT CAC AAT ACC	1341
Met Arg Leu Thr Asn Thr Ala Arg Val Tyr Phe Val Asp His Asn Thr	
355 360 365	
45 AAG ACT ACC ACT TGG GAT GAT CCT CGT TTA CCT TCG TCG TTA GAT CAA	1389
Lys Thr Thr Trp Asp Asp Pro Arg Leu Pro Ser Ser Leu Asp Gln	
370 375 380	
50 AAC GTT CCT CAA TAC AAA CGT GAT TTC CGT AGA AAG TTG ATT TAT TTC	1437
Asn Val Pro Gln Tyr Lys Arg Asp Phe Arg Arg Lys Leu Ile Tyr Phe	
385 390 395 400	
55 CTT TCG CAA CCA GCT TTG CAT CCT TTG CCA GGG CAG TGC CAC ATT AAA	1485
Leu Ser Gln Pro Ala Leu His Pro Leu Pro Gly Gln Cys His Ile Lys	
405 410 415	
60 GTG CGT AGA AAT CAT ATC TTT GAA GAT TCG TAT GCG GAA ATT ATG AGA	1533
Val Arg Arg Asn His Ile Phe Glu Asp Ser Tyr Ala Glu Ile Met Arg	
420 425 430	
65 CAA TCT GCA ACC GAT TTG AAA AAA CGT TTG ATG ATT AAG TTT GAT GGT	1581
Gln Ser Ala Thr Asp Leu Lys Lys Arg Leu Met Ile Lys Phe Asp Gly	
55 435 440 445	
70 GAA GAT GGT TTG GAT TAC CGT GGA TTA TCC CGT GAA TAC TTC TTT TTG	1629
Glu Asp Gly Leu Asp Tyr Gly Gly Leu Ser Arg Glu Tyr Phe Phe Leu	
450 455 460	

5	TTA TCA CAT GAA ATG TTC AAC CCC TTT TAT TGT TTA TTT GAA TAC TCT Leu Ser His Glu Met Phe Asn Pro Phe Tyr Cys Leu Phe Glu Tyr Ser 465 470 475 480	1677
10	TCG GTT GAT AAT TAT ACG CTT CAA ATT AAT CCT CAT TCT GGC ATT AAT Ser Val Asp Asn Tyr Thr Leu Gln Ile Asn Pro His Ser Gly Ile Asn 485 490 495	1725
15	CCA GAG CAT TTG AAC TAT TTC AAG TTC ATA GGC CGA GTC ATT GGT CTC Pro Glu His Leu Asn Tyr Phe Lys Phe Ile Gly Arg Val Ile Gly Leu 500 505 510	1773
20	GCA ATT TTC CAT CGT CCG TTT GTT GAT GCC TTT TTC GTT GTT TCT TTT Ala Ile Phe His Arg Arg Phe Val Asp Ala Phe Val Val Val Ser Phe 515 520 525	1821
25	TAC AAA ATG ATT TTA CAA AAG AAG GTG ACG TTA CAG GAT ATG GAA AGT Tyr Lys Met Ile Leu Gln Lys Val Thr Leu Gln Asp Met Glu Ser 530 535 540	1869
30	ATG GAT GCA GAG TAT TAT CGA AGT TTA GTC TGG ATT TTG GAC AAC GAT Met Asp Ala Glu Tyr Tyr Arg Ser Leu Val Trp Ile Leu Asp Asn Asp 545 550 555 560	1917
35	ATA ACC GGT GTT CTT GAT TTG ACC TTT AGT GTC GAA GAC AAT TGT TTT Ile Thr Gly Val Leu Asp Leu Thr Phe Ser Val Glu Asp Asn Cys Phe 565 570 575	1965
40	GGC GAG GTT GTT ACG ATT GAT TTG AAG CCG AAT GGT CGA AAC ATT GAA Gly Glu Val Val Thr Ile Asp Leu Lys Pro Asn Gly Arg Asn Ile Glu 580 585 590	2013
45	GTT ACA GAA GAG AAT AAA CGC GAA TAT GTT GAT TTG GTG ACT GTA TGG Val Thr Glu Glu Asn Lys Arg Glu Tyr Val Asp Leu Val Thr Val Trp 595 600 605	2061
50	ATT CAA AAA CGT ATA GAA GAG CAG TTT AAT GCA TTT CAT GAA GGT TTT Ile Gln Lys Arg Ile Glu Glu Gln Phe Asn Ala Phe His Glu Gly Phe 610 615 620	2109
55	AGT GAG CTC ATA CCA CAG GAA CTG ATT AAC GTG TTT GAC GAG AGA GAA Ser Glu Leu Ile Pro Gln Glu Leu Ile Asn Val Phe Asp Glu Arg Glu 625 630 635 640	2157
60	TTG GAG TTG TTG ATT GGA GGC ATT TCC GAA ATT GAC ATG GAG GAT TGG Leu Glu Leu Leu Ile Gly Gly Ile Ser Glu Ile Asp Met Glu Asp Trp 645 650 655	2205
65	AAG AAG CAT AAG GAT TAT CGT TCA TAC AGT GAA AAT GAC CAG ATT ATT Lys Lys His Lys Asp Tyr Arg Ser Tyr Ser Glu Asn Asp Gln Ile Ile 660 665 670	2253
70	AAA TGG TTT TGG GAA CTT ATG GAT GAA TGG AGT AAT GAA AAG AAA TCC Lys Trp Phe Trp Glu Leu Met Asp Glu Trp Ser Asn Glu Lys Lys Ser 675 680 685	2301
75	AGA CTT TTA CAA TTT ACC ACT GGT ACT AGC CGA ATT CCT GTC AAC GGG Arg Leu Leu Gln Phe Thr Thr Gly Thr Ser Arg Ile Pro Val Asn Gly	2349

690	695	700		
TTC AAA GAT TTG CAA GGA AGT GAT GGC CCC CGT AAG TTT ACT ATT GAA Phe Lys Asp Leu Gln Gly Ser Asp Gly Pro Arg Lys Phe Thr Ile Glu			2397	
5	705	710	715	
AAA GCT GGT GAA CCC AAT AAA CTT CCC AAG GCC CAC ACC TGT TTC AAT Lys Ala Gly Glu Pro Asn Lys Leu Pro Lys Ala His Thr Cys Phe Asn			2445	
	725	730	735	
10	CGA CTT GAT CTT CCT CCT TAT ACT TCG AAA AAA GAT TTG GAT CAT AAA Arg Leu Asp Leu Pro Pro Tyr Thr Ser Lys Lys Asp Leu Asp His Lys			2493
	740	745	750	
15	TTG TCC ATA GCT GTT GAA GAG ACG ATT GGT TTT GGT CAG GAG Leu Ser Ile Ala Val Glu Glu Thr Ile Gly Phe Gly Gln Glu			2535
	755	760	765	
20	TAAAAATGGAT AGCTAGCTAT TGATTACTTT TGATATTTGA ACTATTGGTG TTTAACAGTG			2595
	AAAAAGAATT TCTGTGTAAA GTTTCCGAAA TTATTTTTTT TTTCTCATTT GAGTGAAAAT			2655
	ACTTGGATCA TCATGTTCTA CCTTTGTGTT CTCTATTACC ATTTTCCTTC TTCTTTTAT			2715
25	ACTTGTTCGC AAACACATTT CCTCTTAATG CTCTTCGCAC AAAACATATA AGTTAATTAA			2775
	CTATTATTAA GTTACGTACT GCATAAGTGA TTTTATATTATGAAATTAC CGCCCTTTT			2835
	CAACATTTTA ATT			2848
30				

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

35 (A) LENGTH: 766 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: protein

40 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

Met	Ser	Asn	Ser	Ala	Gln	Ser	Arg	Arg	Ile	Arg	Val	Ile	Ile	Val	Ala	
1				5					10				15			
45	Ala	Asp	Gly	Leu	Tyr	Lys	Arg	Asp	Val	Phe	Arg	Phe	Pro	Asp	Pro	Phe
				20				25				30				
50	Ala	Val	Leu	Thr	Val	Asp	Gly	Glu	Gln	Thr	His	Thr	Thr	Ala	Ile	
				35				40				45				
	Lys	Lys	Thr	Leu	Asn	Pro	Tyr	Trp	Asn	Glu	Thr	Phe	Glu	Val	Asn	Val
				50				55				60				
55	Thr	Asp	Asn	Ser	Thr	Ile	Ala	Ile	Gln	Val	Phe	Asp	Gln	Lys	Lys	Phe
				65				70			75			80		
	Lys	Lys	Lys	Gly	Gln	Gly	Phe	Leu	Gly	Val	Ile	Asn	Leu	Arg	Val	Gly
				85				90			95					

Asp Val Leu Asp Leu Ala Ile Gly Gly Asp Glu Met Leu Ile Cys Asp
 100 105 110
 5 Leu Lys Lys Leu Asn Glu Asn Thr Val Val His Gly Lys Ile Ile Ile
 115 120 125
 Asn Leu Ser Thr Thr Ala Gln Leu Thr Leu Gln Val Pro Ser Ser Ala
 130 135 140
 10 Ala Ser Gly Ala Arg Thr Gln Arg Thr Ser Ile Thr Asn Asp Pro Gln
 145 150 155 160
 Ser Ser Lys Ser Ser Ser Val Ser Arg Asn Pro Ala Ser Ser Arg Ala
 15 165 170 175
 Gly Ser Pro Thr Arg Asp Asn Ala Pro Ala Ala Ser Pro Ala Ser Ser
 180 185 190
 20 Glu Pro Arg Thr Phe Ser Ser Phe Glu Asp Gln Tyr Gly Arg Leu Pro
 195 200 205
 Pro Gly Trp Glu Arg Cys Thr Asp Asn Leu Gly Arg Thr Tyr Tyr Val
 210 215 220
 25 Asp His Asn Thr Arg Ser Thr Thr Trp Ile Arg Pro Asn Leu Ser Ser
 225 230 235 240
 Val Ala Gly Ala Ala Ala Ala Glu Leu His Ser Ser Ala Ser Ser Ala
 30 245 250 255
 Asn Val Thr Glu Gly Val Gln Pro Ser Ser Ser Asn Ala Ala Arg Arg
 260 265 270
 35 Thr Glu Ala Ser Val Leu Thr Ser Asn Ala Thr Thr Ala Gly Ser Gly
 275 280 285
 Glu Leu Leu Pro Gly Trp Glu Gln Arg Tyr Thr Pro Glu Gly Arg Pro
 290 295 300
 40 Tyr Phe Val Asp His Asn Thr Arg Thr Thr Thr Trp Val Asp Pro Arg
 305 310 315 320
 Arg Gln Gln Tyr Ile Arg Ser Tyr Gly Gly Pro Asn Asn Ala Thr Ile
 45 325 330 335
 Gln Gln Gln Pro Val Ser Gln Leu Gly Pro Leu Pro Ser Gly Trp Glu
 340 345 350
 50 Met Arg Leu Thr Asn Thr Ala Arg Val Tyr Phe Val Asp His Asn Thr
 355 360 365
 Lys Thr Thr Thr Trp Asp Asp Pro Arg Leu Pro Ser Ser Leu Asp Gln
 370 375 380
 55 Asn Val Pro Gln Tyr Lys Arg Asp Phe Arg Arg Lys Leu Ile Tyr Phe
 385 390 395 400
 Leu Ser Gln Pro Ala Leu His Pro Leu Pro Gly Gln Cys His Ile Lys

Lys Ala Gly Glu Pro Asn Lys Leu Pro Lys Ala His Thr Cys Phe Asn
 725 730 735

5 Arg Leu Asp Leu Pro Pro Tyr Thr Ser Lys Lys Asp Leu Asp His Lys
 740 745 750

Leu Ser Ile Ala Val Glu Glu Thr Ile Gly Phe Gly Gln Glu
 755 760 765

10 (2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 3226 base pairs
 (B) TYPE: nucleic acid
 15 (C) STRANDEDNESS: both
 (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: cDNA

20 (ix) FEATURE:
 (A) NAME/KEY: CDS
 (B) LOCATION: 400..2901

25 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CGGTATCAGC AGAGGTGTGT ACGGGCACTG CTTTAAACT GGGAGGAGG AAGACGAGGC 60

30 CAGGGAGCCG GAGGGTCACC AAGGTAGATT TCCAGCAGCG CTAGTCCAGC TGAACACTTT 120

CCAGCCTTGT TTTTCAGCAG CTTTGAGGAA AAGTATAGTG ATCCGTATGT GAAACTTTCA 180

TTGTACGTAG CGGATGAGAA TAGAGAACTT GCTTTGGTCC AGACAAAAAAC ATTAAAAAG 240

35 ACACTGAACC CAAAATGGAA TGAAGAATTIT TATTTCAGGG TAAACCCATC TAATCACAGA 300

CTCCTATTIG AAGTATTGAGA CGAAAATAGA CTGACACGAG ACGGCTTCCT GGGCCAGGTG 360

40 GACGTGCCCG TTAGTCACCT TCCGACAGAA GATCCAACC ATG GAG CGA CCC TAT 414
 Met Glu Arg Pro Tyr
 1 5

45 ACA TTT AAG GAC TTT CTC CTC AGA CCA AGA AGT CAT AAG TCT CGA GTT 462
 Thr Phe Lys Asp Phe Leu Leu Arg Pro Arg Ser His Lys Ser Arg Val
 10 15 20

50 AAG GGA TTT TTG CGA TTG AAA ATG GCC TAT ATG CCA AAA AAT GGA GGT 510
 Lys Gly Phe Leu Arg Leu Lys Met Ala Tyr Met Pro Lys Asn Gly Gly
 25 30 35

55 CAA GAT GAA GAA AAC AGT GAC CAG AGG GAT GAC ATG GAG CAT GGA TGG 558
 Gln Asp Glu Glu Asn Ser Asp Gln Arg Asp Asp Met Glu His Gly Trp
 40 45 50

GAA GTT GTT GAC TCA AAT GAC TCG GCT TCT CAG CAC CAA GAG GAA CTT 606
 Glu Val Val Asp Ser Asn Asp Ser Ala Ser Gln His Gln Glu Glu Leu
 55 60 65

WO 97/12962

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PCT/US96/15930

CCT CCT CCT CCT CTG CCT CCC GGG TGG GAA GAA AAA GTG GAC AAT TTA	654
Pro Pro Pro Pro Leu Pro Pro Gly Trp Glu Glu Lys Val Asp Asn Leu	
70 75 80 85	
5 GGC CGA ACT TAC TAT GTC AAC CAC AAC CGG ACC ACT CAG TGG CAC	702
Gly Arg Thr Tyr Tyr Val Asn His Asn Asn Arg Thr Thr Gln Trp His	
90 95 100	
10 AGA CCA AGC CTG ATG GAC GTG TCC TCG GAG TCG GAC AAT AAC ATC AGA	750
Arg Pro Ser Leu Met Asp Val Ser Ser Glu Ser Asp Asn Asn Ile Arg	
105 110 115	
15 CAG ATC AAC CAG GAG GCA GCA CAC CGG CGC TTC CGC TCC CGC AGG CAC	798
Gln Ile Asn Gln Glu Ala Ala His Arg Arg Phe Arg Ser Arg Arg His	
120 125 130	
20 ATC AGC GAA GAC TTG GAG CCC GAG CCC TCG GAG GGC GGG GAT GTC CCC	846
Ile Ser Glu Asp Leu Glu Pro Glu Pro Ser Glu Gly Gly Asp Val Pro	
135 140 145	
25 GAG CCT TGG GAG ACC ATT TCA GAG GAA GTG AAT ATC GCT GGA GAC TCT	894
Glu Pro Trp Glu Thr Ile Ser Glu Glu Val Asn Ile Ala Gly Asp Ser	
150 155 160 165	
30 CTC GGT CTG GCT CTG CCC CCA CCA CCG GTC TCC CCA GGA TCT CGG ACC	942
Leu Gly Leu Ala Leu Pro Pro Pro Val Ser Pro Gly Ser Arg Thr	
170 175 180	
35 AGC CCT CAG GAG CTG TCA GAG GAA CTA AGC AGA AGG CTT CAG ATC ACT	990
Ser Pro Gln Glu Leu Ser Glu Glu Leu Ser Arg Arg Leu Gln Ile Thr	
185 190 195	
40 CCA GAC TCC AAT GGG GAA CAG TTC AGC TCT TTG ATT CAA AGA GAA CCC	1038
Pro Asp Ser Asn Gly Glu Gln Phe Ser Ser Leu Ile Gln Arg Glu Pro	
200 205 210	
45 TCC TCA AGG TTG AGG TCA TGC AGT GTC ACC GAC GCA GTT GCA GAA CAG	1086
Ser Ser Arg Leu Arg Ser Cys Ser Val Thr Asp Ala Val Ala Glu Gln	
215 220 225	
50 GGC CAT CTA CCA CCG CCA TCA GTG GCC TAT GTA CAT ACC ACG CCG GGT	1134
Gly His Leu Pro Pro Ser Val Ala Tyr Val His Thr Thr Pro Gly	
230 235 240 245	
55 CTG CCT TCA GGC TGG GAA GAA AGA AAA GAT GCT AAG GGG CGC ACA TAC	1182
Leu Pro Ser Gly Trp Glu Glu Arg Lys Asp Ala Lys Gly Arg Thr Tyr	
250 255 260	
60 TAT GTC AAT CAT AAC AAT CGA ACC ACA ACT TGG ACT CGA CCT ATC ATG	1230
Tyr Val Asn His Asn Asn Arg Thr Thr Thr Trp Thr Arg Pro Ile Met	
265 270 275	
65 CAG CTT GCA GAA GAT GGT GCG TCC GGA TCA GCC ACA AAC AGT AAC AAC	1278
Gln Leu Ala Glu Asp Gly Ala Ser Gly Ser Ala Thr Asn Ser Asn Asn	
280 285 290	
70 CAT CTA ATC GAG CCT CAG ATC CGC CGG CCT CGT AGC CTC AGC TCG CCA	1326
His Leu Ile Glu Pro Gln Ile Arg Arg Pro Arg Ser Leu Ser Ser Pro	
295 300 305	

ACA	GTA	ACT	TTA	TCT	GCC	CCG	CTG	GAG	GGT	GCC	AAG	GAC	TCA	CCC	GTA	1374	
Thr	Val	Thr	Leu	Ser	Ala	Pro	Leu	Glu	Gly	Ala	Lys	Asp	Ser	Pro	Val		
310			315												325		
5																	
CGT	CGG	GCT	GTG	AAA	GAC	ACC	CTT	TCC	AAC	CCA	CAG	TCC	CCA	CAG	CCA	1422	
Arg	Arg	Ala	Val	Lys	Asp	Thr	Leu	Ser	Asn	Pro	Gln	Ser	Pro	Gln	Pro		
			330					335							340		
10	TCA	CCT	TAC	AAC	TCC	CCC	AAA	CCA	CAA	CAC	AAA	GTC	ACA	CAG	AGC	TTC	1470
Ser	Pro	Tyr	Asn	Ser	Pro	Lys	Pro	Gln	His	Lys	Val	Thr	Gln	Ser	Phe		
			345				350								355		
15	TTG	CCA	CCC	GGC	TGG	GAA	ATG	AGG	ATA	GCG	CCA	AAC	GGC	CGG	CCC	TTC	1518
Leu	Pro	Pro	Gly	Trp	Glu	Met	Arg	Ile	Ala	Pro	Asn	Gly	Arg	Pro	Phe		
			360			365									370		
20	TTC	ATT	GAT	CAT	AAC	ACA	AAG	ACA	ACA	ACC	TGG	GAA	GAT	CCA	CGT	TTG	1566
Phe	Ile	Asp	His	Asn	Thr	Lys	Thr	Thr	Thr	Trp	Glu	Asp	Pro	Arg	Leu		
			375			380									385		
25	AAA	TTT	CCA	GTA	CAT	ATG	CGG	TCA	AAG	ACA	TCT	TTA	AAC	CCC	AAT	GAC	1614
Lys	Phe	Pro	Val	His	Met	Arg	Ser	Lys	Thr	Ser	Leu	Asn	Pro	Asn	Asp		
			390			395									400	405	
30	CTT	GGC	CCC	CTT	CCT	CCT	GGC	TGG	GAA	GAA	AGA	ATT	CAC	TTG	GAT	GGC	1662
Leu	Gly	Pro	Leu	Pro	Pro	Gly	Trp	Glu	Glu	Arg	Ile	His	Leu	Asp	Gly		
			410			415									420		
35	CGA	ACG	TTT	TAT	ATT	GAT	CAT	AAT	AGC	AAA	ATT	ACT	CAG	TGG	GAA	GAC	1710
Arg	Thr	Phe	Tyr	Ile	Asp	His	Asn	Ser	Lys	Ile	Thr	Gln	Trp	Glu	Asp		
			425			430									435		
40	CCA	AGA	CTG	CAG	AAC	CCA	GCT	ATT	ACT	GGT	CCG	GCT	GTC	CCT	TAC	TCC	1758
Pro	Arg	Leu	Gln	Asn	Pro	Ala	Ile	Thr	Gly	Pro	Ala	Val	Pro	Tyr	Ser		
			440			445									450		
45	AGA	GAA	TTT	AAG	CAG	AAA	TAT	GAC	TAC	TTC	AGG	AAG	AAA	TTA	AAG	AAA	1806
Arg	Glu	Phe	Lys	Gln	Lys	Tyr	Asp	Tyr	Phe	Arg	Lys	Lys	Leu	Lys	Lys		
			455			460									465		
50	CCT	GCT	GAT	ATC	CCC	AAT	AGG	TTT	GAA	ATG	AAA	CTT	CAC	AGA	AAT	AAC	1854
Pro	Ala	Asp	Ile	Pro	Asn	Arg	Phe	Glu	Met	Lys	Leu	His	Arg	Asn	Asn		
			470			475									480	485	
55	ATA	TTT	GAA	GAG	TCC	TAT	CGG	AGA	ATT	ATG	TCC	GTG	AAA	AGA	CCA	GAT	1902
Ile	Phe	Glu	Glu	Ser	Tyr	Arg	Arg	Ile	Met	Ser	Val	Lys	Arg	Pro	Asp		
			490			495									500		
60	GTC	CTA	AAA	GCT	AGA	CTG	TGG	ATT	GAG	TTT	GAA	TCA	GAG	AAA	GGT	CTT	1950
Val	Leu	Lys	Ala	Arg	Leu	Trp	Ile	Glu	Phe	Glu	Ser	Glu	Lys	Gly	Leu		
			505			510									515		
65	GAC	TAT	GGG	GGT	GTG	GCC	AGA	GAA	TGG	TTC	TTC	TTA	CTG	TCC	AAA	GAG	1998
Asp	Tyr	Gly	Gly	Val	Ala	Arg	Glu	Trp	Phe	Phe	Leu	Leu	Ser	Lys	Glu		
			520			525									530		
70	ATG	TTC	AAC	CCC	TAC	TAC	GGC	CTC	TTT	GAG	TAC	TCT	GCC	ACG	GAC	AAC	2046
Met	Phe	Asn	Pro	Tyr	Tyr	Gly	Leu	Phe	Glu	Tyr	Ser	Ala	Thr	Asp	Asn		

	535	540	545	
5	550	555	560	2094
				Tyr Thr Leu Gln Ile Asn Pro Asn Ser Gly Leu Cys Asn Glu Asp His
				565
10	570	575	580	2142
				TTG TCC TAC TTC ACT TTT ATT GGA AGA GTT GCT GGT CTG GCC GTA TTT
				Leu Ser Tyr Phe Thr Phe Ile Gly Arg Val Ala Gly Leu Ala Val Phe
				585
				590
				595
15	600	605	610	2190
				CAT GGG AAG CTC TTA GAT GGT TTC TTC ATT AGA CCA TTT TAC AAG ATG
				His Gly Lys Leu Leu Asp Gly Phe Phe Ile Arg Pro Phe Tyr Lys Met
				585
				590
				595
20	615	620	625	2238
				ATG TTG GGA AAG CAG ATA ACC CTG AAT GAC ATG GAA TCT GTG GAT AGT
				Met Leu Gly Lys Gln Ile Thr Leu Asn Asp Met Glu Ser Val Asp Ser
				600
				605
				610
25	630	635	640	2286
				GAA TAT TAC AAC TCT TTG AAA TGG ATC CTG GAG AAT GAC CCT ACT GAG
				Glu Tyr Tyr Asn Ser Leu Lys Trp Ile Leu Glu Asn Asp Pro Thr Glu
				615
				620
				625
30	640	645	645	2334
				CTG GAC CTC ATG TTC TGC ATA GAC GAA GAA AAC TTT GGA CAG ACA TAT
				Leu Asp Leu Met Phe Cys Ile Asp Glu Glu Asn Phe Gly Gln Thr Tyr
				630
				635
				640
				645
35	650	655	660	2382
				CAA GTG GAT TTG AAG CCC AAT GGG TCA GAA ATA ATG GTC ACA AAT GAA
				Gln Val Asp Leu Lys Pro Asn Gly Ser Glu Ile Met Val Thr Asn Glu
				650
				655
				660
40	665	670	675	2430
				AAC AAA AGG GAA TAT ATC GAC TTA GTC ATC CAG TGG AGA TTT GTG AAC
				Asn Lys Arg Glu Tyr Ile Asp Leu Val Ile Gln Trp Arg Phe Val Asn
				665
				670
				675
45	680	685	690	2478
				AGG GTC CAG AAG CAG ATG AAC GCC TTC TTG GAG GGA TTC ACA GAA CTA
				Arg Val Gln Lys Gln Met Asn Ala Phe Leu Glu Gly Phe Thr Glu Leu
				680
				685
				690
50	695	700	705	2526
				CTT CCT ATT GAT TTG ATT AAA ATT TTT GAT GAA AAT GAG CTG GAG TTG
				Leu Pro Ile Asp Leu Ile Lys Ile Phe Asp Glu Asn Glu Leu Glu Leu
				695
				700
				705
55	710	715	720	2574
				CTC ATG TGC GGC CTC GGT GAT GTG GAT GTG AAT GAC TGG AGA CAG CAT
				Leu Met Cys Gly Leu Gly Asp Val Asp Val Asn Asp Trp Arg Gln His
				710
				715
				720
60	730	735	740	2622
				TCT ATT TAC AAG AAC GGC TAC TGC CCA AAC CAC CCC GTC ATT CAG TGG
				Ser Ile Tyr Lys Asn Gly Tyr Cys Pro Asn His Pro Val Ile Gln Trp
				730
				735
				740
65	745	750	755	2670
				TTC TGG AAG GCT GTG CTA CTC ATG GAC GCC GAA AAG CGT ATC CGG TTA
				Phe Trp Lys Ala Val Leu Leu Met Asp Ala Glu Lys Arg Ile Arg Leu
				745
				750
				755
70	760	765	770	2718
				CTG CAG TTT GTC ACA GGG ACA TCG CGA GTA CCT ATG AAT GGA TTT GCC
				Leu Gln Phe Val Thr Gly Thr Ser Arg Val Pro Met Asn Gly Phe Ala
				760
				765
				770
75	770	775	775	2766
				GAA CTT TAT GGT TCC AAT GGT CCT CAG CTG TTT ACA ATA GAG CAA TGG

Glu Leu Tyr Gly Ser Asn Gly Pro Gln Leu Phe Thr Ile Glu Gln Trp
 775 780 785

5 GGC AGT CCT GAG AAA CTC CCC AGA GCT CAC ACA TGC TTT AAT CGC CTT 2814
 Gly Ser Pro Glu Lys Leu Pro Arg Ala His Thr Cys Phe Asn Arg Leu
 790 795 800 805

10 GAC TTA CCT CCA TAT GAA ACC TTT GAA GAT TTA CGA GAG AAA CTT CTC 2862
 Asp Leu Pro Pro Tyr Glu Thr Phe Glu Asp Leu Arg Glu Lys Leu Leu
 810 815 820

ATG GCC GTG GAA AAT GCT CAA GGA TTT GAA GGG GTG GAT TAAGCACCC 2911
 Met Ala Val Glu Asn Ala Gln Gly Phe Glu Gly Val Asp
 825 830

15 GTGCCTCGGG GGTGGTTGTT CTTCAAGCAA GTTCTGCTTG CACTTTGCA TTTGCCTAAC 2971
 AGACTTTGCA AGAGGCGATG GCAGAGAGCA GCTGCAGGCA TGGTCCCTGG AGCCGAGCCT

20 TCACCA CGCA CTCGTCCAAG TTGGATGCG GGAACCTGGT CCCAGCTTGA GTTCCCTGCCT 3091
 TTCCCACCA AAATTATCAA CTGGTTGATG TGTACACTAA TTACATTCA GGAGGACTTA

25 ATGCTATTTA TGTTGTGCCT CTGCAGCAAA GCCCTTAATA AATATTTAC ATCCTTAAAAA 3211
 AAAAAAAA AAAAA 3226

(2) INFORMATION FOR SEQ ID NO:6:

30 (i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 834 amino acids
 (B) TYPE: amino acid
 (C) TOPOLOGY: linear

35 (ii) MOLECULE TYPE: protein
 (xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

40 Met Glu Arg Pro Tyr Thr Phe Lys Asp Phe Leu Leu Arg Pro Arg Ser
 1 5 10 15

His Lys Ser Arg Val Lys Gly Phe Leu Arg Leu Lys Met Ala Tyr Met
 20 25 30

45 Pro Lys Asn Gly Gly Gln Asp Glu Glu Asn Ser Asp Gln Arg Asp Asp
 35 40 45

50 Met Glu His Gly Trp Glu Val Val Asp Ser Asn Asp Ser Ala Ser Gln
 50 55 60

His Gln Glu Glu Leu Pro Pro Pro Leu Pro Pro Gly Trp Glu Glu
 65 70 75 80

55 Lys Val Asp Asn Leu Gly Arg Thr Tyr Tyr Val Asn His Asn Asn Arg
 85 90 95

Thr Thr Gln Trp His Arg Pro Ser Leu Met Asp Val Ser Ser Glu Ser
 100 105 110

Asp Asn Asn Ile Arg Gln Ile Asn Gln Glu Ala Ala His Arg Arg Phe
 115 120 125

5 Arg Ser Arg Arg His Ile Ser Glu Asp Leu Glu Pro Pro Ser Glu
 130 135 140

Gly Gly Asp Val Pro Glu Pro Trp Glu Thr Ile Ser Glu Glu Val Asn
 145 150 155 160

10 Ile Ala Gly Asp Ser Leu Gly Leu Ala Leu Pro Pro Pro Val Ser
 165 170 175

Pro Gly Ser Arg Thr Ser Pro Gln Glu Leu Ser Glu Glu Leu Ser Arg
 15 180 185 190

Arg Leu Gln Ile Thr Pro Asp Ser Asn Gly Glu Gln Phe Ser Ser Leu
 195 200 205

20 Ile Gln Arg Glu Pro Ser Ser Arg Leu Arg Ser Cys Ser Val Thr Asp
 210 215 220

Ala Val Ala Glu Gln Gly His Leu Pro Pro Pro Ser Val Ala Tyr Val
 225 230 235 240

25 His Thr Thr Pro Gly Leu Pro Ser Gly Trp Glu Glu Arg Lys Asp Ala
 245 250 255

30 Lys Gly Arg Thr Tyr Tyr Val Asn His Asn Asn Arg Thr Thr Thr Trp
 260 265 270

Thr Arg Pro Ile Met Gln Leu Ala Glu Asp Gly Ala Ser Gly Ser Ala
 275 280 285

35 Thr Asn Ser Asn Asn His Leu Ile Glu Pro Gln Ile Arg Arg Pro Arg
 290 295 300

Ser Leu Ser Ser Pro Thr Val Thr Leu Ser Ala Pro Leu Glu Gly Ala
 305 310 315 320

40 Lys Asp Ser Pro Val Arg Arg Ala Val Lys Asp Thr Leu Ser Asn Pro
 325 330 335

45 Gln Ser Pro Gln Pro Ser Pro Tyr Asn Ser Pro Lys Pro Gln His Lys
 340 345 350

Val Thr Gln Ser Phe Leu Pro Pro Gly Trp Glu Met Arg Ile Ala Pro
 355 360 365

50 Asn Gly Arg Pro Phe Phe Ile Asp His Asn Thr Lys Thr Thr Trp
 370 375 380

Glu Asp Pro Arg Leu Lys Phe Pro Val His Met Arg Ser Lys Thr Ser
 385 390 395 400

55 Leu Asn Pro Asn Asp Leu Gly Pro Leu Pro Pro Gly Trp Glu Glu Arg
 405 410 415

Ile His Leu Asp Gly Arg Thr Phe Tyr Ile Asp His Asn Ser Lys Ile

420	425	430
Thr Gln Trp Glu Asp Pro Arg Leu Gln Asn Pro Ala Ile Thr Gly Pro		
435	440	445
Ala Val Pro Tyr Ser Arg Glu Phe Lys Gln Lys Tyr Asp Tyr Phe Arg		
450	455	460
Lys Lys Leu Lys Lys Pro Ala Asp Ile Pro Asn Arg Phe Glu Met Lys		
465	470	475
Leu His Arg Asn Asn Ile Phe Glu Glu Ser Tyr Arg Arg Ile Met Ser		
485	490	495
15 Val Lys Arg Pro Asp Val Leu Lys Ala Arg Leu Trp Ile Glu Phe Glu		
500	505	510
Ser Glu Lys Gly Leu Asp Tyr Gly Gly Val Ala Arg Glu Trp Phe Phe		
515	520	525
20 Leu Leu Ser Lys Glu Met Phe Asn Pro Tyr Tyr Gly Leu Phe Glu Tyr		
530	535	540
Ser Ala Thr Asp Asn Tyr Thr Leu Gln Ile Asn Pro Asn Ser Gly Leu		
545	550	555
25 Cys Asn Glu Asp His Leu Ser Tyr Phe Thr Phe Ile Gly Arg Val Ala		
565	570	575
30 Gly Leu Ala Val Phe His Gly Lys Leu Leu Asp Gly Phe Phe Ile Arg		
580	585	590
Pro Phe Tyr Lys Met Met Leu Gly Lys Gln Ile Thr Leu Asn Asp Met		
595	600	605
35 Glu Ser Val Asp Ser Glu Tyr Tyr Asn Ser Leu Lys Trp Ile Leu Glu		
610	615	620
Asn Asp Pro Thr Glu Leu Asp Leu Met Phe Cys Ile Asp Glu Glu Asn		
625	630	635
40 Phe Gly Gln Thr Tyr Gln Val Asp Leu Lys Pro Asn Gly Ser Glu Ile		
645	650	655
45 Met Val Thr Asn Glu Asn Lys Arg Glu Tyr Ile Asp Leu Val Ile Gln		
660	665	670
Trp Arg Phe Val Asn Arg Val Gln Lys Gln Met Asn Ala Phe Leu Glu		
675	680	685
50 Gly Phe Thr Glu Leu Leu Pro Ile Asp Leu Ile Lys Ile Phe Asp Glu		
690	695	700
Asn Glu Leu Glu Leu Leu Met Cys Gly Leu Gly Asp Val Asp Val Asn		
705	710	715
55 Asp Trp Arg Gln His Ser Ile Tyr Lys Asn Gly Tyr Cys Pro Asn His		
725	730	735

Pro Val Ile Gln Trp Phe Trp Lys Ala Val Leu Leu Met Asp Ala Glu
 740 745 750

5 Lys Arg Ile Arg Leu Leu Gln Phe Val Thr Gly Thr Ser Arg Val Pro
 755 760 765

Met Asn Gly Phe Ala Glu Leu Tyr Gly Ser Asn Gly Pro Gln Leu Phe
 770 775 780

10 Thr Ile Glu Gln Trp Gly Ser Pro Glu Lys Leu Pro Arg Ala His Thr
 785 790 795 800

Cys Phe Asn Arg Leu Asp Leu Pro Pro Tyr Glu Thr Phe Glu Asp Leu
 805 810 815

15 Arg Glu Lys Leu Leu Met Ala Val Glu Asn Ala Gln Gly Phe Glu Gly
 820 825 830

20 Val Asp

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

25 (A) LENGTH: 2415 base pairs
 (B) TYPE: nucleic acid
 (C) STRANDEDNESS: both
 (D) TOPOLOGY: linear

30 (ii) MOLECULE TYPE: cDNA

(ix) FEATURE:

35 (A) NAME/KEY: CDS
 (B) LOCATION: 1..2412

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

40 ATG GTG TCC TCT GGC CTC ACT GAA AAC CAG CTA CAG CTC TCT GTA GAG 48
 Met Val Ser Ser Gly Leu Thr Glu Asn Gln Leu Gln Leu Ser Val Glu
 1 5 10 15

45 GTG TTG ACA TCC CAC TCT TGT TCT GAG GAA GGC TTA GAG GAT GCA GCC 96
 Val Leu Thr Ser His Ser Cys Ser Glu Glu Gly Leu Glu Asp Ala Ala
 20 25 30

50 AAC GTA CTA CTG CAG CTC TCC CGG GGG GAC TCT GGG ACC CGG GAC ACT 144
 Asn Val Leu Leu Gln Leu Ser Arg Gly Asp Ser Gly Thr Arg Asp Thr
 35 40 45

55 GTT CTC AAG CTG CTA CTG AAT GGA GCC CGC CAT CTG GGT TAT ACC CTT 192
 Val Leu Lys Leu Leu Asn Gly Ala Arg His Leu Gly Tyr Thr Leu
 50 55 60

TGT AAA CAA ATA GGT ACC CTG CTG GCC GAG CTG CGG GAA TAC AAC CTC 240
 Cys Lys Gln Ile Gly Thr Leu Leu Ala Glu Leu Arg Glu Tyr Asn Leu
 65 70 75 80

GAG CAG CAG CGG CGA GCC CAA TGT GAA ACC CTC TCT CCT GAT GGC CTG			288	
Glu Gln Gln Arg Arg Ala Gln Cys Glu Thr Leu Ser Pro Asp Gly Leu				
	85	90	95	
5	CCT GAG GAG CAG CCA CAG ACC ACC AAG CTG AAG GGC AAA ATG CAG AGC			336
	Pro Glu Glu Gln Pro Gln Thr Thr Lys Leu Lys Gly Lys Met Gln Ser			
	100	105	110	
10	AGG TTT GAC ATG GCT GAG AAT GTG GTA ATT GTG GCA TCT CAG AAG CGA			384
	Arg Phe Asp Met Ala Glu Asn Val Val Ile Val Ala Ser Gln Lys Arg			
	115	120	125	
15	CTT TTG GGT GGC CGG GAG CTC CAG CTG CCT TCT ATG TCC ATG TTG ACA			432
	Leu Leu Gly Gly Arg Glu Leu Gln Leu Pro Ser Met Ser Met Leu Thr			
	130	135	140	
	TCC AAG ACA TCT ACC CAG AAG TTC TTC TTG AGG GTA CTA CAG GTC ATC			480
	Ser Lys Thr Ser Thr Gln Lys Phe Phe Leu Arg Val Leu Gln Val Ile			
	145	150	155	160
20	ATC CAG CTC CGG GAC GAC ACG CGC CGG GCT AAC AAG AAA GCC AAG CAG			528
	Ile Gln Leu Arg Asp Asp Thr Arg Arg Ala Asn Lys Lys Ala Lys Gln			
	165	170	175	
25	ACA GGC AGG CTA GGT TCC TCC GGT TTA GGC TCA GCT AGC AGC ATC CAG			576
	Thr Gly Arg Leu Gly Ser Ser Gly Leu Gly Ser Ala Ser Ser Ile Gln			
	180	185	190	
30	GCA GCT GTT CGG CAG CTG GAG GCT GAT GCC ATT ATA CAA ATG			624
	Ala Ala Val Arg Gln Leu Glu Ala Glu Ala Asp Ala Ile Ile Gln Met			
	195	200	205	
35	GTA CGT GAG GGT CAA AGG GCG CGG AGA CAG CAA CAA GCA GCA ACG TCG			672
	Val Arg Glu Gly Gln Arg Ala Arg Arg Gln Gln Ala Ala Thr Ser			
	210	215	220	
	GAG TCT AGC CAG TCA GAG GCG TCT GTC CGG AGG GAG GAA TCA CCC ATG			720
	Glu Ser Ser Gln Ser Glu Ala Ser Val Arg Arg Glu Glu Ser Pro Met			
	225	230	235	240
40	GAT GTG GAC CAG CCA TCT CCC AGT GCT CAA GAT ACT CAA TCC ATT GCC			768
	Asp Val Asp Gln Pro Ser Pro Ser Ala Gln Asp Thr Gln Ser Ile Ala			
	245	250	255	
45	TCC GAT GGA ACC CCA CAG GGG GAG AAG GAA AAG GAA GAA AGA CCA CCT			816
	Ser Asp Gly Thr Pro Gln Gly Glu Lys Glu Lys Glu Glu Arg Pro Pro			
	260	265	270	
50	GAG TTA CCC CTG CTC AGC GAG CAG CTG AGT TTG GAC GAG CTG TGG GAC			864
	Glu Leu Pro Leu Leu Ser Glu Gln Leu Ser Leu Asp Glu Leu Trp Asp			
	275	280	285	
55	ATG CTT GGG GAG TGT CTA AAG GAA CTA GAG GAA TCC CAT GAC CAG CAT			912
	Met Leu Gly Glu Cys Leu Lys Glu Leu Glu Ser His Asp Gln His			
	290	295	300	
	GCG GTG CTA GTG CTA CAG CCT GCT GTC GAG GCC TTC TTT CTG GTC CAT			960
	Ala Val Leu Val Leu Gln Pro Ala Val Glu Ala Phe Phe Leu Val His			
	305	310	315	320

	GCC ACA GAG CGG GAG AGC AAG CCT CCT GTC CGA GAC ACC CGT GAG AGC Ala Thr Glu Arg Glu Ser Lys Pro Pro Val Arg Asp Thr Arg Glu Ser 325 330 335	1008
5	CAG CTG GCA CAC ATC AAG GAC GAG CCT CCT CTA CTC TCC CCT GCC CCC Gln Leu Ala His Ile Lys Asp Glu Pro Pro Leu Leu Ser Pro Ala Pro 340 345 350	1056
10	TTA ACC CCA GCC ACG CCT TCC TCC CTT GAC CCA TTC TTC TCC CGG GAG Leu Thr Pro Ala Thr Pro Ser Ser Leu Asp Pro Phe Phe Ser Arg Glu 355 360 365	1104
15	CCC TCA TCT ATG CAC ATC TCC TCA AGC CTG CCC CCT GAC ACA CAG AAG Pro Ser Ser Met His Ile Ser Ser Ser Leu Pro Pro Asp Thr Gln Lys 370 375 380	1152
20	TTC CTT CGC TTT GCA GAG ACT CAC CGC ACT GTG TTA AAC CAG ATC CTA Phe Leu Arg Phe Ala Glu Thr His Arg Thr Val Leu Asn Gln Ile Leu 385 390 395 400	1200
25	CGG CAG TCC ACG ACC CAC CTT GCT GAT GGG CCT TTT GCT GTC CTG GTA Arg Gln Ser Thr Thr His Leu Ala Asp Gly Pro Phe Ala Val Leu Val 405 410 415	1248
30	GAC TAC ATT CGT GTC CTC GAC TTT GAT GTC AAG CGC AAA TAT TTC CGC Asp Tyr Ile Arg Val Leu Asp Phe Asp Val Lys Arg Lys Tyr Phe Arg 420 425 430	1296
35	CAA GAG CTG GAG CGT TTA GAT GAG GGG CTC CGG AAA GAA GAC ATG GCT Gln Glu Leu Glu Arg Leu Asp Glu Gly Leu Arg Lys Glu Asp Met Ala 435 440 445	1344
40	GTG CAT GTC CGT GAC CAT GTG TTT GAA GAC TCC TAT CGT GAG CTG Val His Val Arg Arg Asp His Val Phe Glu Asp Ser Tyr Arg Glu Leu 450 455 460	1392
45	CAT CGC AAA TCC CCC GAA GAA ATG AAG AAT CGA TTG TAT ATA GTA TTT His Arg Lys Ser Pro Glu Glu Met Lys Asn Arg Leu Tyr Ile Val Phe 465 470 475 480	1440
50	GAA GGA GAA GAA GGG CAG GAT GCT GGC GGG CTC CTG CGG GAG TGG TAT Glu Gly Glu Gly Gln Asp Ala Gly Gly Leu Leu Arg Glu Trp Tyr 485 490 495	1488
55	ATG ATC ATC TCT CGA GAG ATG TTT AAC CCT ATG TAT GCC TTG TTC CGT Met Ile Ile Ser Arg Glu Met Phe Asn Pro Met Tyr Ala Leu Phe Arg 500 505 510	1536
55	ACC TCA CCT GGT GAT CGA GTC ACC TAC ACC AAT CCA TTT TCC CAC Thr Ser Pro Gly Asp Arg Val Thr Tyr Thr Ile Asn Pro Phe Ser His 515 520 525	1584
55	TGC AAC CCC AAC CAC CTC AGC TAC TTC AAG TTT GTC GGA CGC ATT GTG Cys Asn Pro Asn His Leu Ser Tyr Phe Lys Phe Val Gly Arg Ile Val 530 535 540	1632
	GCC AAA GCT GTA TAT GAC AAC CGT CTT CTG GAG TGC TAC TTT ACT CGA Ala Lys Ala Val Tyr Asp Asn Arg Leu Leu Glu Cys Tyr Phe Thr Arg	1680

545	550	555	560	
5	TCC TTT TAC AAA CAC ATC TTG GGC AAG TCA GTC AGA TAT ACA GAT ATG Ser Phe Tyr Lys His Ile Leu Gly Lys Ser Val Arg Tyr Thr Asp Met 565	570	575	1728
10	GAG AGT GAA GAT TAC CAC TTC TAC CAA GGT CTG GTT TAT CTG CTG GAA Glu Ser Glu Asp Tyr His Phe Tyr Gln Gly Leu Val Tyr Leu Leu Glu 580	585	590	1776
15	AAT GAT GTC TCC ACA CTA GGC TAT GAC CTC ACC TTC AGC ACT GAG GTC Asn Asp Val Ser Thr Leu Gly Tyr Asp Leu Thr Phe Ser Thr Glu Val 595	600	605	1824
20	CAA GAG TTT GGA GTT TGT GAA GTT CGT GAC CTC AAA CCC AAT GGG GCC Gln Glu Phe Gly Val Cys Glu Val Arg Asp Leu Lys Pro Asn Gly Ala 610	615	620	1872
25	AAC ATC TTG GTA ACA GAG GAG AAT AAG AAG GAG TAT GTA CAC CTG GTA Asn Ile Leu Val Thr Glu Glu Asn Lys Lys Glu Tyr Val His Leu Val 625	630	635	1920
30	TGC CAG ATG AGA ATG ACA GGA GCC ATC CGC AAG CAG TTG GCG GCT TTC Cys Gln Met Arg Met Thr Gly Ala Ile Arg Lys Gln Leu Ala Ala Phe 645	650	655	1968
35	TTA GAA GGC TTC TAT GAG ATC ATT CCA AAG CGC CTC ATT TCC ATC TTC Leu Glu Gly Phe Tyr Glu Ile Ile Pro Lys Arg Leu Ile Ser Ile Phe 660	665	670	2016
40	ACT GAG CAG GAG TTA GAG CTG CTT ATA TCA GGA CTG CCC ACC ATT GAC Thr Glu Gln Glu Leu Leu Ile Ser Gly Leu Pro Thr Ile Asp 675	680	685	2064
45	ATC GAT GAT CTG AAA TCC AAC ACT GAA TAC CAC AAG TAC CAG TCC AAC Ile Asp Asp Leu Lys Ser Asn Thr Glu Tyr His Lys Tyr Gln Ser Asn 690	695	700	2112
50	TCT ATT CAG ATC CAG TGG TTC TGG AGA GCA TTG CGT TCT TTC GAT CAA Ser Ile Gln Ile Gln Trp Phe Trp Arg Ala Leu Arg Ser Phe Asp Gln 705	710	715	2160
55	GCT GAC CGT GCC AAG TTC CTC CAG TTT GTC ACG GGT ACT TCC AAG GTA Ala Asp Arg Ala Lys Phe Leu Gln Phe Val Thr Gly Thr Ser Lys Val 725	730	735	2208
60	CCC CTG CAA GGC TTT GCT GCC CTC GAA GGC ATG AAT GGC ATT CAG AAG Pro Leu Gln Gly Phe Ala Ala Leu Glu Gly Met Asn Gly Ile Gln Lys 740	745	750	2256
65	TTT CAG ATC CAT CGA GAT GAC AGG TCC ACA GAT CGC CTG CCT TCA GCT Phe Gln Ile His Arg Asp Asp Arg Ser Thr Asp Arg Leu Pro Ser Ala 755	760	765	2304
70	CAC ACA TGT TTT AAT CAG CTG GAT CTG CCT GCC TAT GAG AGC TTT GAG His Thr Cys Phe Asn Gln Leu Asp Leu Pro Ala Tyr Glu Ser Phe Glu 770	775	780	2352
75	AAG CTC CGC CAC ATG CTA CTG TTG GCT ATC CAG GAG TGC TCT GAA GGC			2400

Lys Leu Arg His Met Leu Leu Leu Ala Ile Gln Glu Cys Ser Glu Gly
 785 790 795 800

2415

5 TTT GGG CTG GCC TAA
 Phe Gly Leu Ala

(2) INFORMATION FOR SEQ ID NO:8:

10

(i) SEQUENCE CHARACTERISTICS:
 (A) LENGTH: 804 amino acids
 (B) TYPE: amino acid
 (D) TOPOLOGY: linear

15

(ii) MOLECULE TYPE: protein

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

20 Met Val Ser Ser Gly Leu Thr Glu Asn Gln Leu Gln Leu Ser Val Glu
 1 5 10 15

Val Leu Thr Ser His Ser Cys Ser Glu Glu Gly Leu Glu Asp Ala Ala
 20 25 30

25 Asn Val Leu Leu Gln Leu Ser Arg Gly Asp Ser Gly Thr Arg Asp Thr
 35 40 45

30 Val Leu Lys Leu Leu Asn Gly Ala Arg His Leu Gly Tyr Thr Leu
 50 55 60

Cys Lys Gln Ile Gly Thr Leu Leu Ala Glu Leu Arg Glu Tyr Asn Leu
 65 70 75 80

35 Glu Gln Gln Arg Arg Ala Gln Cys Glu Thr Leu Ser Pro Asp Gly Leu
 85 90 95

40 Pro Glu Glu Gln Pro Gln Thr Thr Lys Leu Lys Gly Lys Met Gln Ser
 100 105 110

Arg Phe Asp Met Ala Glu Asn Val Val Ile Val Ala Ser Gln Lys Arg
 115 120 125

45 Leu Leu Gly Gly Arg Glu Leu Gln Leu Pro Ser Met Ser Met Leu Thr
 130 135 140

Ser Lys Thr Ser Thr Gln Lys Phe Phe Leu Arg Val Leu Gln Val Ile
 145 150 155 160

50 Ile Gln Leu Arg Asp Asp Thr Arg Arg Ala Asn Lys Lys Ala Lys Gln
 165 170 175

Thr Gly Arg Leu Gly Ser Ser Gly Leu Gly Ser Ala Ser Ser Ile Gln
 180 185 190

55 Ala Ala Val Arg Gln Leu Glu Ala Glu Ala Asp Ala Ile Ile Gln Met
 195 200 205

Val Arg Glu Gly Gln Arg Ala Arg Arg Gln Gln Ala Ala Thr Ser

-99-

210 215 220

Glu Ser Ser Gln Ser Glu Ala Ser Val Arg Arg Glu Glu Ser Pro Met
225 230 235 240

5

Asp Val Asp Gln Pro Ser Pro Ser Ala Gln Asp Thr Gln Ser Ile Ala
245 250 25510 Ser Asp Gly Thr Pro Gln Gly Glu Lys Glu Lys Glu Glu Arg Pro Pro
260 265 270Glu Leu Pro Leu Leu Ser Glu Gln Leu Ser Leu Asp Glu Leu Trp Asp
275 280 28515 Met Leu Gly Glu Cys Leu Lys Glu Leu Glu Glu Ser His Asp Gln His
290 295 300Ala Val Leu Val Leu Gln Pro Ala Val Glu Ala Phe Phe Leu Val His
305 310 315 32020 Ala Thr Glu Arg Glu Ser Lys Pro Pro Val Arg Asp Thr Arg Glu Ser
325 330 33525 Gln Leu Ala His Ile Lys Asp Glu Pro Pro Leu Leu Ser Pro Ala Pro
340 345 350Leu Thr Pro Ala Thr Pro Ser Ser Leu Asp Pro Phe Phe Ser Arg Glu
355 360 36530 Pro Ser Ser Met His Ile Ser Ser Ser Leu Pro Pro Asp Thr Gln Lys
370 375 380Phe Leu Arg Phe Ala Glu Thr His Arg Thr Val Leu Asn Gln Ile Leu
385 390 395 40035 Arg Gln Ser Thr Thr His Leu Ala Asp Gly Pro Phe Ala Val Leu Val
405 410 41540 Asp Tyr Ile Arg Val Leu Asp Phe Asp Val Lys Arg Lys Tyr Phe Arg
420 425 430Gln Glu Leu Glu Arg Leu Asp Glu Gly Leu Arg Lys Glu Asp Met Ala
435 440 44545 Val His Val Arg Arg Asp His Val Phe Glu Asp Ser Tyr Arg Glu Leu
450 455 460His Arg Lys Ser Pro Glu Glu Met Lys Asn Arg Leu Tyr Ile Val Phe
465 470 475 48050 Glu Gly Glu Glu Gly Gln Asp Ala Gly Gly Leu Leu Arg Glu Trp Tyr
485 490 49555 Met Ile Ile Ser Arg Glu Met Phe Asn Pro Met Tyr Ala Leu Phe Arg
500 505 510Thr Ser Pro Gly Asp Arg Val Thr Tyr Thr Ile Asn Pro Phe Ser His
515 520 525

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-100-

Cys Asn Pro Asn His Leu Ser Tyr Phe Lys Phe Val Gly Arg Ile Val
 530 535 540

Ala Lys Ala Val Tyr Asp Asn Arg Leu Leu Glu Cys Tyr Phe Thr Arg
 5 545 550 555 560

Ser Phe Tyr Lys His Ile Leu Gly Lys Ser Val Arg Tyr Thr Asp Met
 565 570 575

10 Glu Ser Glu Asp Tyr His Phe Tyr Gln Gly Leu Val Tyr Leu Leu Glu
 580 585 590

Asn Asp Val Ser Thr Leu Gly Tyr Asp Leu Thr Phe Ser Thr Glu Val
 595 600 605

15 Gln Glu Phe Gly Val Cys Glu Val Arg Asp Leu Lys Pro Asn Gly Ala
 610 615 620

Asn Ile Leu Val Thr Glu Glu Asn Lys Lys Glu Tyr Val His Leu Val
 20 625 630 635 640

Cys Gln Met Arg Met Thr Gly Ala Ile Arg Lys Gln Leu Ala Ala Phe
 645 650 655

25 Leu Glu Gly Phe Tyr Glu Ile Ile Pro Lys Arg Leu Ile Ser Ile Phe
 660 665 670

Thr Glu Gln Glu Leu Glu Leu Ile Ser Gly Leu Pro Thr Ile Asp
 675 680 685

30 Ile Asp Asp Leu Lys Ser Asn Thr Glu Tyr His Lys Tyr Gln Ser Asn
 690 695 700

Ser Ile Gln Ile Gln Trp Phe Trp Arg Ala Leu Arg Ser Phe Asp Gln
 35 705 710 715 720

Ala Asp Arg Ala Lys Phe Leu Gln Phe Val Thr Gly Thr Ser Lys Val
 725 730 735

40 Pro Leu Gln Gly Phe Ala Ala Leu Glu Gly Met Asn Gly Ile Gln Lys
 740 745 750

Phe Gln Ile His Arg Asp Asp Arg Ser Thr Asp Arg Leu Pro Ser Ala
 755 760 765

45 His Thr Cys Phe Asn Gln Leu Asp Leu Pro Ala Tyr Glu Ser Phe Glu
 770 775 780

Lys Leu Arg His Met Leu Leu Leu Ala Ile Gln Glu Cys Ser Glu Gly
 50 785 790 795 800

Phe Gly Leu Ala

We claim:

1. An isolated and/or recombinant *pub* polypeptide, which polypeptide comprises an amino acid sequence identical or homologous to the amino acid sequence designated by one or more of SEQ ID No. 2, SEQ ID No. 4, SEQ ID No. 6 and/or SEQ ID No. 8, or a fragment thereof.
2. The *pub* polypeptide of claim 1, which polypeptide affects cell-cycle progression of eukaryotic cells.
3. The *pub* polypeptide of claim 1, which polypeptide possesses a ubiquitin ligase activity.
4. The *pub* polypeptide of claim 3, which polypeptide ubiquitinates cdc25, p53 or both.
5. The *pub* polypeptide of claim 1, which polypeptide is at least 75% homologous to amino acid sequence designated by SEQ ID NO. 2.
6. The *pub* polypeptide of claim 1, which polypeptide is at least 75% homologous to amino acid sequence designated by SEQ ID NO. 4.
7. The *pub* polypeptide of claim 1, which polypeptide is at least 75% homologous to amino acid sequence designated by SEQ ID NO. 6.
8. The *pub* polypeptide of claim 1, which polypeptide is at least 75% homologous to amino acid sequence designated by SEQ ID NO. 8.
9. The *pub* polypeptide of claim 1, which polypeptide is a fusion protein.
10. The *pub* polypeptide of claim 1, which polypeptide is of mammalian origin.
11. The *pub* polypeptide of claim 9, which polypeptide is of human origin.
- 35 12. The *pub* polypeptide of claim 1, which polypeptide is encoded by a nucleic acid which hybridizes under stringent conditions to at least a portion of the nucleic acid designated by SEQ ID No. 1 corresponding to a catalytic domain, a calcium lipid binding domain, or both.

13. The *pub* polypeptide of claim 1, which polypeptide is encoded by a nucleic acid which hybridizes under stringent conditions to at least a portion of the nucleic acid designated by SEQ ID No. 5 or SEQ ID No. 7 corresponding to a catalytic domain.
- 5 14. A isolated nucleic acid comprising a nucleotide sequence encoding a *pub* polypeptide, or a nucleotide sequence complementary thereto, said *pub* polypeptide including an amino acid sequence identical or homologous to the amino acid sequence designated by one or more of SEQ ID No. 2, SEQ ID No. 4, SEQ ID No. 6 and/or SEQ ID No. 8, or a portion thereof.
- 10 15. The nucleic acid of claim 14, wherein said *pub* polypeptide possesses a ubiquitin ligase activity.
- 15 16. The nucleic acid of claim 14, wherein said *pub* polypeptide possesses a calcium binding motif.
17. The nucleic acid of claim 14, wherein said *pub* polypeptide ubiquitinates cdc25, p53 or both.
- 20 18. The nucleic acid of claim 14, wherein said *pub* polypeptide encoding nucleotide sequence is at least 75% homologous to amino acid sequence designated by SEQ ID NO. 1.
- 25 19. The nucleic acid of claim 14, wherein said *pub* polypeptide encoding nucleotide sequence is at least 75% homologous to amino acid sequence designated by SEQ ID NO. 3.
- 30 20. The nucleic acid of claim 14, wherein said *pub* polypeptide encoding nucleotide sequence is at least 75% homologous to amino acid sequence designated by SEQ ID NO. 5.
- 35 21. The nucleic acid of claim 14, wherein said *pub* polypeptide encoding nucleotide sequence is at least 75% homologous to amino acid sequence designated by SEQ ID NO. 7.
22. The nucleic acid of claim 14, wherein said *pub* polypeptide is a fusion protein.
23. The nucleic acid of claim 14, which nucleic acid hybridizes under stringent conditions to a nucleic acid probe having a sequence represented by at least 60

consecutive nucleotides of SEQ ID No. 1, 3, 5 and/or 7, or a sequence complementary thereto.

24. The nucleic acid of claim 14, wherein said *pub* polypeptide encoding nucleotide sequence hybridizes under stringent conditions to a nucleic acid probe having a sequence represented by at least 60 consecutive nucleotides of SEQ ID No. 1, 3, 5 and/or 7, or a sequence complementary thereto.
- 5 25. The nucleic acid of claim 14, further comprising a transcriptional regulatory sequence operably linked to said nucleotide sequence so as to render said nucleic acid suitable for use as an expression vector.
- 10 26. An expression vector, capable of replicating in at least one of a prokaryotic cell and eukaryotic cell, comprising the nucleic acid of claim 25.
- 15 27. A host cell transfected with the expression vector of claim 26 and expressing said recombinant polypeptide.
- 20 28. A method of producing a recombinant *pub* polypeptide comprising culturing the cell of claim 27 in a cell culture medium to express said recombinant polypeptide and isolating said recombinant polypeptide from said cell culture.
- 25 29. A transgenic animal having cells which harbor a transgene comprising the nucleic acid of claim 14, or in which a gene comprising said nucleic acid is disrupted.
- 30 30. Isolated nucleic acid which selectively hybridizes under high stringency conditions to at least ten nucleotides of a nucleic acid sequence represented by one of SEQ ID Nos. 1, 3, 5 and/or 7, or complementary sequences thereof, which nucleic acid can specifically detect or amplify a nucleic acid sequence of a *pub* gene .
31. The nucleic acid of claim 30, which is nucleic acid is labelled.
32. An assay for identifying an agent which modulates ubiquitination of a protein, comprising:
 - 35 (i) providing a ubiquitin-conjugating system which comprises a *pub* ligase, a substrate protein which undergoes ubiquitination by the *pub* ligase, and ubiquitin, under conditions which promote *pub*-dependent ubiquitination of the substrate protein;
 - (ii) contacting the ubiquitin-conjugating system with a candidate agent;

5 (iii) measuring a level of ubiquitination of the substrate protein in the presence of the candidate agent; and
(iv) comparing the measured level of ubiquitination in the presence of the candidate agent with a level of ubiquitination of the substrate protein in the absence of the candidate agent,

wherein a statistically significant change in ubiquitination of the substrate protein in the presence of the candidate agent is indicative of an agent which modulates ubiquitination of the substrate protein.

10 33. The assay of claim 32, wherein the ubiquitin-conjugating system is a reconstituted protein mixture.

34. The assay of claim 32, wherein the ubiquitin-conjugating system is a cell lysate.

15 35. The assay of claim 32, wherein the ubiquitin-conjugating system is a cell expressing a recombinant *pub* ligase.

36. The assay of claim 32, wherein the *pub* ligase is a mammalian *pub* protein.

20 37. The assay of claim 32, wherein the *pub* ligase is a recombinant polypeptide.

38. The assay of claim 32, wherein the substrate protein is a cdc25 phosphatase.

25 39. The assay of claim 32, wherein the ubiquitin is provided in a form selected from a group consisting of:
(i) an unconjugated ubiquitin, in which case the ubiquitin-conjugating system further comprises an E1 ubiquitin-activating enzyme (E1), an E2 ubiquitin-conjugating enzyme (E2), and adenosine triphosphate;

30 (ii) an activated E1:ubiquitin complex, in which case the ubiquitin-conjugating system further comprises an E2;

(iii) an activated E2:ubiquitin complex; and

(iv) an activated *pub*:ubiquitin complex.

40. An assay for identifying an agent which competitively inhibits binding of a *pub* ubiquitinating complex with a protein, comprising:
35 (i) forming a mixture comprising a *pub* polypeptide, a substrate protein which undergoes ubiquitination by the *pub* polypeptide, and a candidate agent;
(ii) measuring a level of binding between the substrate protein and the *pub* polypeptide in the presence of the candidate agent; and

(iv) comparing the measured level of binding in the presence of the candidate agent with a level of binding of the substrate protein to the *pub* polypeptide in the absence of the candidate agent,

wherein a statistically significant decrease in binding of the substrate protein to the *pub* polypeptide in the presence of the candidate agent is indicative of an agent which competitively inhibits binding of a *pub* polypeptide with the substrate protein.

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41. The *pub* polypeptide of claim 1, which polypeptide is encoded by a nucleic acid which hybridizes under stringent conditions to at least a portion of the nucleic acid designated by SEQ ID No. 3 corresponding to a catalytic domain, a calcium lipid binding domain, or both.

INTERNATIONAL SEARCH REPORT

Int'l. Application No	PCT/US 96/15930
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A. CLASSIFICATION OF SUBJECT MATTER
 IPC 6 C12N9/00 C12Q1/25

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
 IPC 6 C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	<p>NATURE, vol. 373, 5 January 1995, LONDON, GB, pages 81-83, XP000611565 M. SCHEFFNER ET AL.: "Protein ubiquitination involving an E1-E2-E3 enzyme ubiquitin thioester cascade" cited in the application see the whole document</p> <p>---</p> <p style="text-align: center;">-/-</p>	1-6, 32-39

Further documents are listed in the continuation of box C.

Patent family members are listed in annex.

* Special categories of cited documents :

- *A* document defining the general state of the art which is not considered to be of particular relevance
- *E* earlier document but published on or after the international filing date
- *L* document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
- *O* document referring to an oral disclosure, use, exhibition or other means
- *P* document published prior to the international filing date but later than the priority date claimed

- *T* later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
- *X* document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
- *Y* document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.
- *&* document member of the same patent family

1

Date of the actual completion of the international search

22 January 1997

Date of mailing of the international search report

31.01.97

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 European Patent Office, P.B. 5818 Patentstaan 2
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 Fax (+ 31-70) 340-3016

Authorized officer

Mateo Rosell, A.M.

INTERNATIONAL SEARCH REPORT

Intern	Application No
PCT/US 96/15930	

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES, vol. 92, - 1 March 1995 WASHINGTON,US, pages 2563-2567, XP000611567 J.M. HUIBREGTSE ET AL.: "A family of proteins structurally and functionally related to the E6-AP ubiquitin-protein ligase" cited in the application see the whole document ---	1-6, 14-28
X	MOLECULAR AND CELLULAR BIOLOGY, vol. 13, no. 2, - 1 February 1993 WASHINGTON,US, pages 775-784, XP000602210 HUIBREGTSE ET AL.: "Cloning and expression of the cDNA for E6-AP, a protein that mediates the interaction of the human papillomavirus E6 oncoprotein with p53" see figures 3,AND,9 ---	1-6
X	BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS, vol. 185, no. 3, 30 June 1992, FLORIDA,US, pages 1155-1161, XP000611569 S. KUMAR ET AL.: "Identification of a set of genes with developmentally down-regulated expression in the mouse brain" cited in the application see the whole document ---	1-7, 13-28, 30,31
X	MOLECULAR BRAIN RESEARCH, vol. 24, - 1994 AMSTERDAM,NL, pages 77-88, XP000196792 J. GU ET AL: "Cloning of a DNA binding protein that is a tyrosine kinase substrate and recognizes an upstream initiator-like sequence in the promoter of the preprodynorphin gene" figure 3 see the whole document ---	1-5,8, 14-28
X	WO 95 18974 A (MITOTIX INC) 13 July 1995 see abstract ---	40
P,X	EMBO JOURNAL, vol. 15, no. 6, 1996, OXFORD,GB, pages 1301-1312, XP000616164 B. NEFSKY AND D. BEACH: "Pub1 acts as an E6-AP-like protein ubiquitin ligase in the degradation of cdc25" see the whole document -----	12,19, 23,24, 30,31

INTERNATIONAL SEARCH REPORT

Information on patent family members

Inter Application No
PCT/US 96/15930

Patent document cited in search report	Publication date	Patent family member(s)		Publication date
WO-A-9518974	13-07-95	AU-A-	1866995	01-08-95